Intraspecies Diversity of *Candida albicans*: Genetic and Functional Studies

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I can.
I will.

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1 Summary

In health the fungus *Candida albicans* resides as a harmless member of the normal human microbiota on mucosal surfaces and is tolerated by the immune system. However, when surrounding conditions change the host is unable to control fungal colonization. This enables *C. albicans* to switch from commensal to pathogenic state. Consequently the majority of infections arise from the colonizing fungi, but also nosocomial transmissions are possible.

Candida species are the most frequent fungal commensals on the buccal mucosa and skin. After all, prevalence of *Candida* colonization depends on the investigated population and geographical region. Colonization rates appeared to be overestimated for the investigated region of southern Switzerland, but *C. albicans* remained the most prevalent species. *C. albicans* isolates from healthy and diseased individuals displayed an extensive genetic diversity, but neither distinct alleles nor clonal complexes could be correlated with the origin of the isolates.

Furthermore we focused on the interaction of randomly selected natural isolates with the host, especially at mucosal sites, in the well-established model of oropharyngeal candidiasis. The isolates displayed no gross differences in growth or cell wall composition *in vitro*, but they triggered highly variable degrees of inflammation *in vivo*. High-pathogenic isolates induced a rapid and acute immune response leading to fungal clearance. Whereas, weak and delayed induction of Interleukin 17 (IL-17) and antimicrobial effector molecules by certain isolates correlated with their persistence in the mucosal epithelium. IL-17-mediated immunity has emerged as a critical mechanism of the host to regulate the antimicrobial response, thereby limiting fungal overgrowth at the epithelial barriers. This is conserved among *C. albicans* isolates, irrespective of the underlying pathogenicity. Complementarily, neutrophils contribute to host defense by preventing dissemination of highly pathogenic fungi. Albeit low-pathogenic persistent isolates do not induce neutrophil recruitment. The underlying mechanism of immune evasion of low-pathogenic isolates remains to be elucidated. In a co-infection with a low- and high-pathogenic isolate we demonstrated the persistence of the low-pathogenic isolate despite the induction of a strong inflammatory response by the high-pathogenic isolate. The induced immune response led to the selective clearing of the high-pathogenic isolate irrespective of the close co-localization of both isolates at the same areas of the tongue.

It is generally believed that the host immune status alone determines the outcome of the interaction between the commensal fungus and its host, resulting in either health or disease. This study demonstrates that the natural diversity of *C. albicans* comes into the limelight for the fine balance between commensalism and disease *in vivo*.

2 Zusammenfassung

Bei gesunden Individuen weilt der Pilz *Candida albicans* als harmloses Mitglied des normalen menschlichen Mikrobioms auf mukösen Oberflächen und wird vom Immunsystem toleriert. Ändern sich allerdings die Bedingungen, ist der Wirt nicht mehr in der Lage die Pilz-Kolonisierung zu kontrollieren. Dieser Umstand ermöglicht es *C. albicans* vom kommensalen in den pathogenen Zustand zu wechseln. Folglich werden die meisten Infektionen vom kolonisierenden Pilz hervorgerufen, obgleich auch nosokomiale Übertragungen möglich sind.

Candida Spezies sind die häufigsten kommensalen Pilze in der Mundschleimhaut und auf der Haut. Nichtsdestotrotz ist die Häufigkeit der Kolonisierung mit *Candida* abhängig von der untersuchten Population und dem geographischen Gebiet. Die Kolonisierungsrate scheint für die untersuchte Region der Südschweiz überbewertet zu sein, allerdings bleibt *C. albicans* die vorherrschende Spezies. *C. albicans* Isolate von gesunden und erkrankten Individuen zeigten eine sehr hohe genetische Diversität. Jedoch konnte keine Korrelation zwischen bestimmten Allelen oder klonalen Gruppen und der Herkunft der Isolate festgestellt werden.

Des Weiteren haben wir die Interaktion zwischen zufällig ausgewählten klinischen Isolaten und dem Wirt untersucht, im Speziellen mit der Mukosa im bewährten oropharyngealen Candidiasis Model. Die Isolate zeigten keine massiven Unterschiede im Wachstum oder der Zusammensetzung der Zellwand *in vitro*, aber sie lösten *in vivo* stark unterschiedliche Grade der Inflammation aus. Hoch-pathogene Isolate induzierten eine schnelle und starke Immunantwort, welche zur kompletten Beseitigung des Pilzes führte. Wohingegen eine schwache und verspätete Induktion von Interleukin 17 (IL-17) und antimikrobiellen Effektor-Molekülen durch bestimmte Isolate mit der Persistenz im mukosalen Epithelium korrelierte.

IL-17-vermittelte Immunität hat sich als entscheidender Wirtsmechanismus herausgestellt, welcher die antimikrobielle Antwort reguliert und dabei das Überwachsen des Pilzes an der Epithel-Barriere

limitiert. Dieser Mechanismus besteht konserviert bei allen Isolaten, unabhängig von ihrer Pathogenität. Komplementär tragen Neutrophile zur Wirtsantwort bei, indem sie die Ausbreitung von hoch-pathogenen Pilzen verhindern. Obgleich schwach pathogene Isolate keine Rekrutierung der Neutrophilen hervorrufen. Der zugrundeliegende Mechanismus der Immunevasion von schwach-pathogenen Isolaten muss noch genauer erforscht werden. In einer Ko-Infektion mit einem schwach- und einem hoch-pathogenen Isolat, konnten wir die Persistent des schwach-pathogenen Isolates zeigen, trotz der starken Immunantwort durch das hoch-pathogene Isolat. Die induzierte Immunantwort führte zu einer selektiven Beseitigung des hoch-pathogenen Isolates ungeachtet der nahen Ko-Lokalisation von beiden Isolaten an der gleichen Stelle der Zunge.

Es wird allgemein angenommen, dass nur das Immunsystem des Wirtes den Ausgang der Interaktion zwischen dem kommensalen Pilz und dem Wirt bestimmt, was folglich zu Gesundheit oder Krankheit führt. Diese Studie zeigt deutlich, dass die natürliche Diversität von *C. albicans* an Bedeutung für das empfindliche Gleichgewicht zwischen Kommensalismus und Krankheit *in vivo* gewinnt.

3 General Introduction

3.1 *Candida albicans*: from harmless commensal to pathogen

In a world full of microbes, 1.5 million different fungal species are known (Butler 2010). Fungi appear in a broad range of shape, size and structure. They occur in the soil, on plants, animals, humans and in extreme environments such as hyperosmotic areas or the desert (Vaupotic et al. 2008). Fungi are also used in our daily life: to brew beer, make wine or raise bread dough. In addition to the use in food industry, fungi are also indispensable in the pharmaceutical industry. (Demain, Sanchez 2009). A majority of fungi positively contribute to people´s life and well' being and only a few hundred fungi are actually pathogenic for humans (Butler 2010).

Those pathogenic species include *Apergillus fumigatus*, *Histoplasma capsulatum* and *Cryptococcus neoformans*, which live as environmental fungi, but can cause exogenous infections in humans (Casadevall 2008). However, most human fungal pathogens cause endogenous infections. They live as part of the normal microbiota on the epithelial surface of healthy individuals and only cause disease symptoms if natural barriers are breached. *Malassezia* and *Candida* are part of this group with an adaptation to an endogenous life (Hube 2009).

The species of *Candida* belong to the phylus of ascomycetes and can be found both in human and other mammalian sources and also, to a lesser extent, in the environment (McManus, Coleman 2014). Only a few members are of clinical importance. Among those are *Candida dubliniesnis*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata* and *Candida albicans* (Pfaller 1996; Pfaller et al. 2007). The most pathogenic and prevalent one is *C. albicans*, the 2nd most common nosocomial infection agent in the US (Mendes Giannini, M. J. S. et al. 2013; Delaloye, Calandra 2014).

3.1.1 Polymorphism

C. albicans is a polymorphic fungus, meaning it can switch between several morphological states (Odds 1988). Only *C. albicans* and its closest relative *C. dubliniensis* can form true hyphae, and they can grow in the yeast and filamentous form (Jackson et al. 2009).

The three main phenotypes of *C. albicans* are the yeast form (formerly called blastospores), pseudohyphae and true hyphae (Berman, Sudbery 2002) (Figure 1). Hyphae and pseudohyphae are classified as filamentous morphologies because of their growth in an elongated form. Pseudohyphae look similar to hyphae but are physiologically closer to yeast cells (Odds 1988; Sudbery et al. 2004).

Pseudohyphal cells are usually ellipsoid and show a constriction at the septin ring (Sudbery 2001). In contrast, hyphal cells have parallel sides and true septa with pores for a cell-cell communication, which is non−existent in pseudohyphae (Odds 1988). In nature, a mix and/or intermediates of all morphological forms are found, such as parallel-sided pseudohyphae or elongated single yeast cells (Thompson et al. 2011).

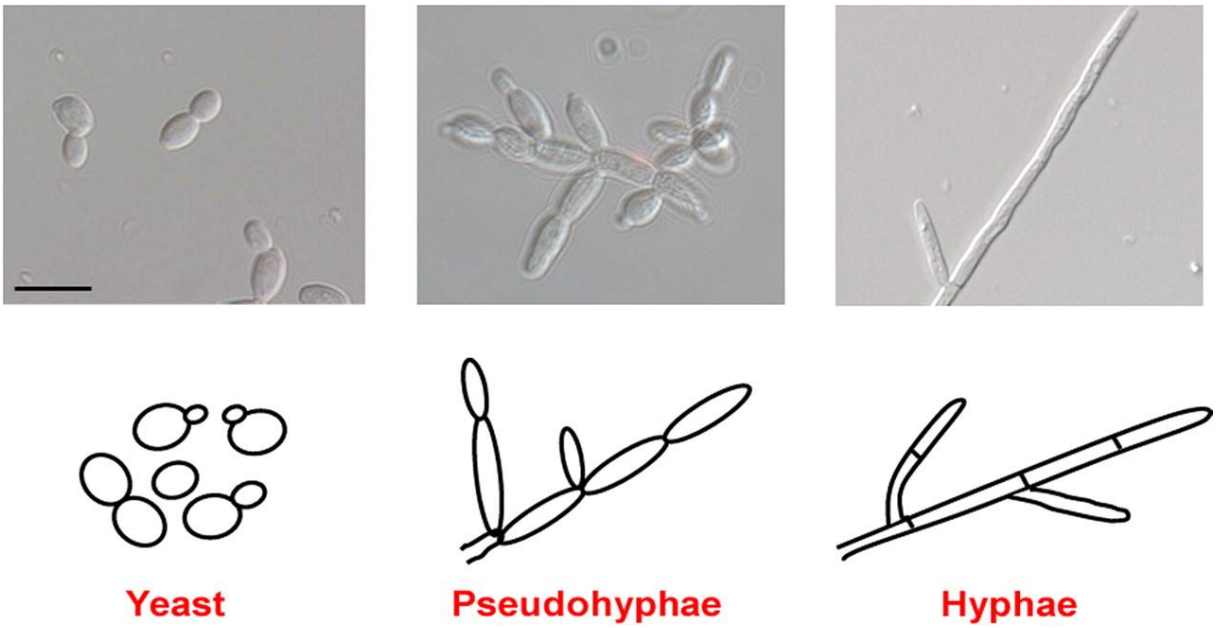

Figure 1. Morphological forms of *C. albicans*. (Top) Differential interference contrast microscopy (black bar 10 µm). (Bottom) schematic picture of each morphology (Thompson et al. 2011)

It is generally believed that the yeast form appears during non-invasive commensalism and that, on the contrary, the hyphal form of the fungus is more invasive (Berman, Sudbery 2002). However *in vivo* studies revealed the expression of hyphae-associated genes, such as *HWP1*, during both commensalism and disease (Naglik, JR et al. 2006). In fact, both forms are found in infected organs (Odds 1988). It is proposed that during dissemination in the bloodstream, yeast cells are the predominant form due to their shape and size (Jacobsen et al. 2012). Serum components and blood are stimuli for hyphae formation, a hyphae to yeast switch after breaking the first epithelial barrier therefore seems rather unlikely. It is proposed that hyphae cells provide access for yeast cells to the underlying tissue and bloodstream (Jacobsen et al. 2012). A mechanism of contact sensing, or thigmotropism, allows hyphal cells to recognize and penetrate weak point of the host tissue (Gow et al. 2002).

Recently novel morphological phenotypes have been discovered. The white and opaque morphologies are part of a tristable phenotype switching system including the gray phenotype (Tao et al. 2014). The

phenotypic switch between the white and opaque form correlate with mating and biofilm formation (Miller, Johnson 2002; Daniels et al. 2006). They show differences in terms of: activity of the secreted aspartyl protease (SAP), colony morphology, mating competency and virulence (Tao et al. 2014).

3.1.2 Virulence factors

C. albicans evolved several factors which contribute to the pathogenic potential (Mayer et al. 2013). Among them are various mechanisms of interaction with epithelial cells including adherence, invasion and induction of damage (Zhu, Filler 2010). Additionally, yeast-to-hyphae transition, secretion of hydrolytic enzymes and fast adaptation to environmental changes, helps C. albicans to colonize diverse host niches (Mayer et al. 2013). A fast adaptation is also facilitated by the stress response to heat, oxidative, nitrosative or osmotic stress (Haynes et al. 2012).

3.1.2.1 Adherence and Biofilm

A basic requirement of the interaction between *C. albicans* and the host surfaces is adherence to epithelial cells. Therefore adherence to abiotic surfaces (e.g. catheters) or biotic surfaces (e.g. epithelial cells) are the first steps of colonization, growth and development of disease (Zakikhany et al. 2007; Wachtler et al. 2011b). Hyphae formation and adhesion are closely related. After contact with host cells or abiotic surfaces, hyphae-associated adhesion genes are highly expressed (Jacobsen et al. 2012). Adherence, and subsequent colonization, is also a known risk factor for disseminated candidiasis (Marr et al. 2000). *C. albicans* has different mechanisms of adherence and expresses different surface structures (Zhu, Filler 2010). The family of agglutinin-like surface structures (Als) consist of eight glycosylphosphatidylinositol (GPI)-anchored surface proteins that mediate adherence and invasion to host substrates (Zhao et al. 2005; Sheppard et al. 2004). The hyphal wall protein (Hwp1) is only expressed on hyphal cells and binds to proteins on the host surface by functionally mimicking host proteins (Ponniah et al. 2007).

Hwp1 also binds to Als1 and Als3 and mediates cell-cell contact, which is especially important for biofilm formation (Nobile et al. 2008). Biofilm on medical devices counts as risk factor for systemic candidiasis as dispersed yeast cells from the biofilm can disseminate into the bloodstream (Uppuluri et al. 2010).

3.1.2.2 Invasion

A further key event of the *C. albicans* pathogenesis is cell invasion. In a murine model of mucosal candidiasis it was shown that *C. albicans* with a reduced ability to invade causes a less severe infection (Naglik, JR et al. 2008). Invasion into epithelial cells can occur through two distinct mechanisms: host induced endocytosis or fungal driven active penetration (Dalle et al. 2010; Wachtler et al. 2011b). The

interaction between Als3, a fungal invasin and E-cadherin or Human epidermal growth factor receptor 2 (Her2) drives induced endocytosis. Her2 also forms a heterodimer with the epidermal growth factor receptor (EGFR) and leads to clathrin-dependent uptake of the fungus (Phan et al. 2007; Moreno-Ruiz et al. 2009; Zhu, Filler 2010). This usually occurs within the first hours after initial contact (Tang et al. 2016). Als3 is expressed by hyphal cells, consequently endocytosis has only been observed for *C. albicans* hyphae (Phan et al. 2007; Zakikhany et al. 2007). Induced endocytosis is exclusively host driven, because inactivated hyphae are taken up in a similar manner than viable hyphae (Tang et al. 2016). In contrast, active penetration is fungi−dependent; hyphal extension and consequential expression of SAPs are thus important factors (Naglik, JR et al. 2003a; Wachtler et al. 2011b).

3.1.2.3 Damage

During superficial candidiasis, damage is defined as the loss and permeabilization of the superficial epithelium (Farah et al. 2000). The mechanism of damage induction is not completely understood, but SAPs are known to play an important role. A large number of genes associated with damage is also involved in thigmotropism (Wachtler et al. 2011b). The capacity to induce damage is a feature of mucosal pathogens which distinguishes them from harmless commensals (Koropatnick et al. 2004).

Recently, the first fungal cytolytic peptide toxin has been discovered. The secreted peptide, Candidalysin (Ece1), damages epithelial membranes probably by membrane permeabilization and calcium influx. Next to tissue damage, Ece1 induces epithelial immunity (Moyes et al. 2016).

It has been shown that interepithelial penetration following the initial invasion might play a major role in damage induction by the fungus (Zakikhany et al. 2007). Beside the fungus, the inflammatory cells infiltrating in the infected tissue may lead to a substantial tissue damage (Wallach et al. 2014).

3.1.3 Diversity of *C. albicans* isolates

Natural isolates of *C. albicans* exhibit a high molecular diversity, at the level of chromosome rearrangements as well as single nucleotides (Afsarian et al. 2015; Selmecki et al. 2010).The variability in the karyotype of clinical isolates is still poorly understood (Rustchenko 2007; Merz et al. 1988; Rustchenko-Bulgac 1991). Genomic changes can be: loss of heterozygosity (LOH), aneuploidy and general chromosomal rearrangements or chromosome truncations (Rustchenko 2007; Selmecki et al. 2010). Multiple repeat sequences are the favorable sites of translocation and are found on all chromosomes, except chromosome 3 (Chindamporn et al. 1998). It is suggested that extreme genomic changes reveal a fast adaptation mechanism to different stressors (Selmecki et al. 2010). It is most likely that this genomic rearrangements contribute to high variation in the phenotype. A single passage

through the mouse host increase the variability in phenotype and leads to genomic rearrangements (Forche et al. 2005;Forche et al. 2009) .

Based on variations on the genome level, a large spectrum of phenotypic attributes across isolates have been observed. Clinical isolates show quantitative variation in their ability to form biofilm (Li et al. 2003) as well as stress and antifungal resistance, growth rates and filamentation (Hirakawa et al. 2015). Furthermore, striking intraspecies differences in virulence were found in a murine model of systemic infection (MacCallum et al. 2009).

3.1.4 Types of *Candida* infections

Vertical and ,to a lesser extent, horizontal transmission contribute to *Candida* colonization (Bliss et al. 2008). Colonization is defined by a harmless co-existence with the host in equilibrium with the microbiome (Jacobsen et al. 2012). Most cases of candidiasis are caused by an endogenous source, such as *C. albicans* , which is a commensal in the oral cavity (Pfaller 1996; Hattori et al. 2006; Shimizu et al. 2011). The appearance of *C. albicans* in the host can be differentiated in colonization, superficial infection and systemic infection (Odds 1994).

3.1.4.1 Superficial infections

3.1.4.1.1 Vulvovaginal candidiasis

Half of all woman experience vulvovaginal candidiasis (VVC) at least once during the course of their life. Approximately 5 % of these woman develop recurrent episodes of vulvovaginal candidiasis (RVVC) (Mardh et al. 2002). Pregnancy and estrogen replacement in postmenopausal woman increase the risk of RVVC (Geiger A. M., Foxman 1996). Superficial infections are usually caused by a disturbance of the normal microbial flora or a compromised immune system. Vaginal candidiasis and symptomless colonization are equally common in healthy and HIV−positive woman (Schuman et al. 1998). This indicates that, in contrast to other *Candida* infections, vaginitis is not related to immunosupression, but rather to external predisposing factors like dysbiosis and antibiotic treatment (Mardh et al. 2002)

3.1.4.1.2 Oral candidiasis

Oral candidiasis is the most common oral infection in humans (Muzyka 2005). Among the local risk factors are denture wearing, poor mouth hygiene, intake of broad spectrum antibiotic and a high sugar diet. Several local but more likely systemic factors are known to increase the risk of developing the disease. A reduced immune status, like HIV infection, endocrine disease, chemotherapy and immunosuppressive drugs are part of systemic factors, with possibility of one patient presenting several of those factors simultaneously (Darwazeh, Darwazeh 2014). As opposed to vaginal candidiasis,

oropharyngeal candidiasis occurs in 80-90 % of HIV−positive patients (Samaranayake 1992). Consequently it is also referred as ¨the disease of the diseased¨ (Darwazeh, Darwazeh 2014).

3.1.4.1.3 Chronic mucocutaneous candidiasis

Chronic mucocutaneous candidiasis (CMC) is described as a heterologous group of disorders with a persistent, non-invasive *Candida* infection of the skin, nails and mucosa (Heimall et al. 2010; Puel et al. 2011). In contrast to other superficial *Candida* infections, the underlying cause of disease is often genetic and not environmental. CMC is caused by acquired T-cell immunodeficiencies or inborn errors of immunity (Figure 2) (Milner, Holland 2013b). We will further concentrate on primary immundeficiencies underlying CMC.

During an infection *C. albicans* invades and is recognized via Dectin 1, Dectin-2 by dendritic cells (DCs). This triggers, through the spleen tyrosine kinase (Syk), the expression of proinflammatory cytokines IL-6, IL-23 and tumor necrosis factor (TNF-α) mediated by caspase recruitment domain family member (CARD9) (Plato et al. 2013).

IL-17A and IL-17F induction by T-cells and innate lymphoid cells requires IL-23 production by DCs. (Hirota et al. 2011; Kashem et al. 2015a). Signal transducer and activator of transcription 3 (STAT3) and STAT1 are required for the induction of Th17 cells.

Patients with mutations in the activator of transcription 3 (*STAT3)*, *STAT1* and *CARD9* display an impaired development of T-cells, which produce IL-17 and IL-22 (Milner et al. 2008; Minegishi et al. 2009; Glocker et al. 2009). Patients affected by autosomal recessive autoimmune polyendocrinopathy syndrome (APECED) have mutations in *AIRE,* which leads to high titers of antibodies against IL-17A, IL-17F and IL-22 (Husebye et al. 2009). The central role of IL-17 in fungal immunity is promoted by the identification of mutations in IL-17F and IL-17RA in CMC patients (Puel et al. 2011).

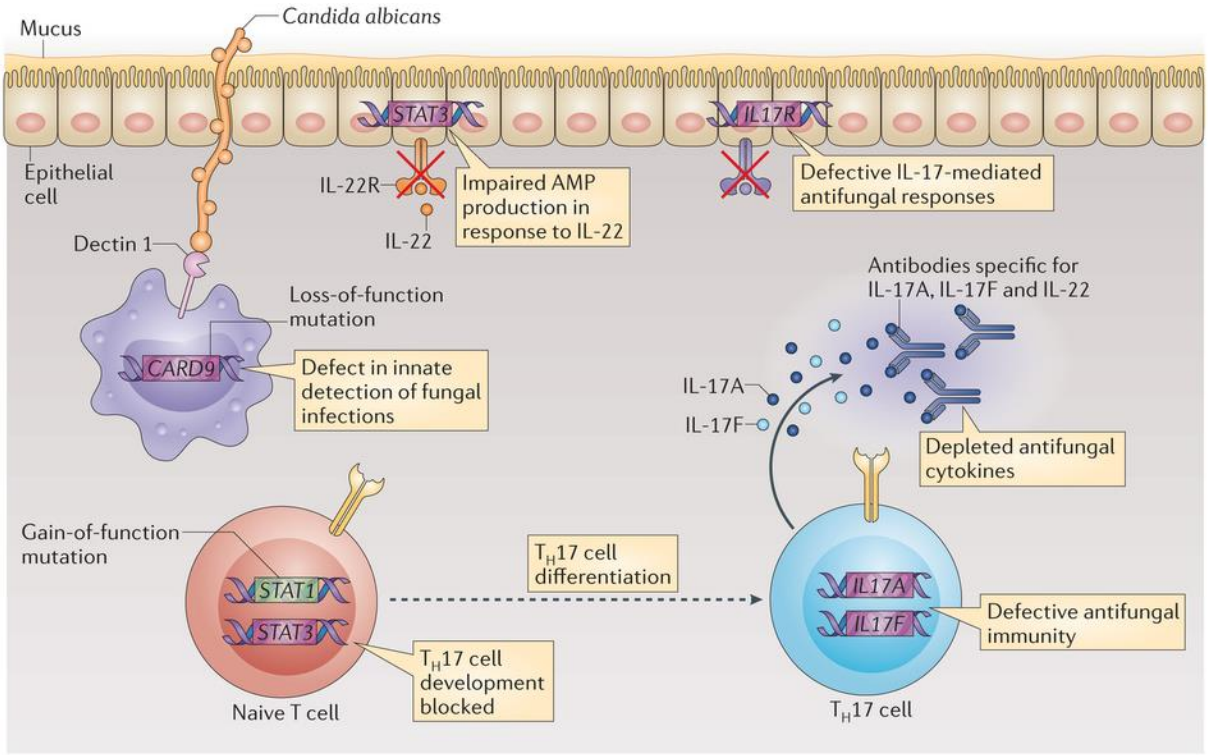

Figure 2 Defects in IL-17-IL17 receptor pathway cause CMC. *C. albicans* invade and are recognized via Dectin1 and 2 by dendritic cells. Mutation in *CARD9* leads to an impaired detection of the fungus. A gain-of-function mutation in *STAT1* and dominant-negative mutation in *STAT3* blocks the induction of Th17 cells and in case of *STAT3* to an impaired production of AMP. Mutations in *IL17A/F* or *IL17R* and antibodies against IL-17A, IL-17F and IL-22 results in a defective antifungal immunity. Dashed arrows indicate T-cell differentiation (Milner, Holland 2013b).

3.1.4.2 Systemic infections

Systemic infections with *C. albicans* are characterized by dissemination of the fungus in the bloodstream. *C. albicans* has two different routes to enter the bloodstream, either by gastrointestinal or skin-derived candidiasis (Lionakis 2014). Entry through the gastrointestinal mucosal barrier is exclusively seen in immunosuppressed patients, usually in combination with other risk factors. Skin-derived candidiasis arises in patients at the intensive care unit by breached integrity of the skin e.g. via intravenous catheters or open wounds (Kullberg, Am Oude 2002; Lionakis 2014). Leading risk factors are thus central venous catheter, immunosupression, neutropenia and gastrointestinal surgery and burns (Perlroth et al. 2007; Lionakis 2014). Candidemia can be a manifestation of invasive candidiasis originating from various organs (Fridkin 2005).

3.2 Host response to *C. albicans* infections

The immune system consists of three main components: the physical barrier, the innate and adaptive immune system.

3.2.1 Epithelial recognition and innate immune response

The first line of defense is the epithelium, in some tissues in combination with a mucus layer and/or the commensal flora, as a physical barrier and through the regulation of a cytokine and antimicrobial peptide (AMP) network. Epithelial cells can discriminate between commensal and pathogenic forms of *Candida*, due to the fungal load and the morphology. This enables them to orchestrate the innate immunity (Moyes et al. 2010). The nuclear factor NF-κB and the initial mitogen-activated protein kinase (MAPK) response are independent of fungal morphology (Moyes et al. 2010). In contrast, the activation of the c-Fos transcription factor and the MAPK phosphatase MKP1 through a secondary MAPK response in epithelial cells is hyphae−dependent (Moyes et al. 2010). The cytolytic peptide toxin Ece1, called Candidalysin, was shown to be necessary and sufficient to induce epithelial immune activation (Moyes et al. 2016). OPC infection with *ece1Δ/Δ* lacking the cytolytic peptide fragment 3 is unable to induce damage, phosphorylation of MKP1 and activation of c-Fos (Moyes et al. 2016). Fungal recognition by epithelial cells is less well understood than the mechanism of fungal recognition by myeloid cells, such as macrophages and monocytes.

The initial step in innate immune activation is the recognition of fungal pathogen-associated molecular patterns (PAMP) by pathogen recognition receptors (PRR). PAMP on the surface of *C. albicans* include mannans, beta-glucans and chitin, which can be recognized by the mannose receptor, dectin-1, dectin-2, Toll-like receptor 2 (TLR2), TLR4 and the complement receptor 3 (CR3) (Jouault et al. 2009; Netea et al. 2008). Fungal recognition by epithelial cells is likely not via the classical PAMPS and PRRs, rather through interaction with E-cadherin and Her2 (Moyes et al. 2010; Zhu et al. 2012; Naglik et al. 2014)

Human oral epithelial cells release IL-1α, IL-1β, Granulocyte-macrophage colony-stimulating factor (GM-CSF), Granulocyte colony-stimulating factor (G-CSF), IL-6 and “regulated on activation normal T cell expressed and secreted” (RANTES) (Weindl et al. 2010).

The resulting signal transduction leads to the release of pro-inflammatory cytokines/chemokines, which further promotes the activation of the adaptive immune system, if PRR signaling takes place in DCs/APCs (LeibundGut-Landmann et al. 2007; Tang et al. 2016). These cytokines/chemokines in turn trigger an inflammatory response and recruit inflammatory immune cells including neutrophils and monocytes to the site of infection. Neutrophils were reported to enhance the production of

antimicrobial peptides and alarmins, like Calprotectin (S100A8/9) and beta-defensins (Weindl et al. 2010; Moyes, Naglik 2011).

3.2.1.1 Neutrophil and macrophage mediated antifungal defense

After invasion, *C. albicans* face phagocytic cells. Mobilization of neutrophils from the bone-marrow occurs in a G-CSF dependent manner (Roberts 2005). Neutrophils are recruited into the infected tissue by locally produced chemokines. Following the invasion of *Candida*, keratinocytes release IL-1α which elevates the production of G-CSF (Altmeier et al. 2016). Macrophages and recruited neutrophils can both phagocytose yeast and short hyphae cells. Neutrophils can inhibit the growth, the yeast-to hyphae transition and efficiently kill the fungus (Fradin et al. 2005). After oral infection, neutrophils prevent dissemination into deeper tissue layer (Brown 2011).

Phagocytosed yeast cells may escape by transforming into hyphae and killing the macrophage (Lorenz et al. 2004). Phagocytic cells kill *C. albicans* by the production of reactive oxygen species, a mechanism also called oxidative burst. The fungus produces superoxide dismutase to escape this attack (Fradin et al. 2005; Frohner et al. 2009). Hyphae cells which can not be engulfed are killed or inhibited by the release of neutrophil extracellular traps (NETs), also mast cells and eosinophils can cope with microbes by extracellular traps (Urban et al. 2009). NETs contain fibers of released DNA and histones with antimicrobial effector molecules, like Calprotectin (Urban et al. 2009).

3.2.1.2 Antimicrobial peptides

Epithelial cells, neutrophils and other immune cells can produce and secrete diverse antimicrobial peptides (Kiyoura, Tamai 2015). Cathelicidin (LL-37) acts against bacterial and viral skin infection (Lopez-Garcia et al. 2005). In *C. albicans* it causes an efflux of proteins and nucleotides by a massive disruption of the cell membrane (den Hertog et al. 2005). It was also demonstrated to be chemotactic for monocytes and neutrophils (Kiyoura, Tamai 2015).

In humans, six β-defensins have been identified and all of them can be found in oral epithelial cells and the saliva (Diamond et al. 2001). In an oral *Candida* model, murine β-defensins (mBD1) plays a role in early fungal control and the induction of innate inflammatory modulators (Tomalka et al. 2015). mBD3 shows strong similarity with the hBD2 and serves as an innate defense against oral *Candida* infections (Burd et al. 2002).

Histatin 5 (Hst-5) is the most potent antimicrobial peptide in the saliva (Puri et al. 2015). Compared to LL-37, it causes smaller leaks in the cell membrane (den Hertog et al. 2005).

In several infection models, Calprotectin is critically for protection from C. albicans (Urban et al. 2009; Trautwein-Weidner et al. 2015b). We focused on S100A9, a subunit of the Calprotectin protein complex, which chelates calcium and zinc. It was also found to be attached to NETs and to be essential for antifungal activity *in vitro* (Urban et al. 2009).

3.2.2 Adaptive immune response:

PRRs on antigen presenting cells (APCs) bind PAMPs, like beta-glucan and mannan, and phagocytose the fungus. Three distinct signals are essential for the priming of T-cells. Antigenic peptides are presented by MHC class II lead in combination with APC-mediated co-stimulation to activation and expansion of T-cells. The cytokine milieu, depending on the induced signals by PRRs, direct the T cell polarization and associated effector functions. In consequence, T cells differentiate into different subsets (Th1, Th2, Th17, regulatory Tcells (Treg)), each with a characteristic effector function (Zhu et al. 2010; Richardson, Moyes 2015). *Candida* specific T-cells are the Interferon-γ secreting Th1 cells and IL-17 secreting Th17 cells (Kashem et al. 2015b). During Th17 differentiation, cells might become Tregs, determined by the cytokine milieu (Ghoreschi et al. 2010). The Th2 phenotype is rather associated with outgrowth and dissemination of the fungus (Richardson, Moyes 2015). The importance of T cells in the antifungal immunity is highlighted by the high prevalence of mucocutaneous candidiasis in AIDS patients with $CD4^+$ T-cell depletion (Fidel, JR 2011).

3.2.2.1 Non-redundant role of IL-17

The family of the IL-17 cytokines consists of six members (IL-17A-IL-17F) and five receptors (IL-17RA-IL-17RE) (Gaffen 2009). The hallmark cytokines IL-17A and IL-17F (called IL-17 from hereon, if not specified otherwise), can form hetero- and homodimers and signal through the same heteromeric receptor complex IL-17RA-IL-17RC (Wright et al. 2008). IL-17 can be secreted by a variety of cells including Th cells and innate lymphoid cells (ILC) (Cua, Tato 2010; Gladiator et al. 2013). Epithelial cells and fibroblasts are the main targets of IL-17 (Gaffen 2009). IL-17 is strictly required for fungal control and host protection. IL-17 mediated mechanisms are critical to eliminate the fungus from the host tissue, presumably by the regulation of antimicrobial peptides. In a murine model of oropharyngeal candidiasis (OPC), IL-17 is not required for neutrophil recruitment (Trautwein-Weidner et al. 2015b), even though this function of IL-17 was demonstrated in other settings. In addition to T cells, innate sources of IL-17 have been described in mouse models of acute candidiasis (cutaneous candidiasis, OPC) (Gladiator et al. 2013; Sparber, LeibundGut-Landmann 2015; Kashem et al. 2015a)

4 Aim of this thesis

C. albicans is an important opportunistic pathogen in humans. Disease symptoms occur if specific protective immune mechanisms are breached. Antifungal immune defense mechanisms that protect from different forms of diseases have been characterized. It is generally believed that the host immune status determines the outcome of the interaction between the commensal fungus and its host. In addition to host factors, the intrinsically high diversity of C. albicans favors health or disease.

This work sets out to investigate the natural diversity among *C. albicans* isolates and the impact on the decision between health and disease.

In the first project, we assess the colonization rates by *Candida* spp. on the oral mucosae and skin of healthy children in southern part of Switzerland. Prevalence of colonization fluctuates depending on investigated age and geographical region. Furthermore, we compare isolates from healthy individuals by MLMT analysis with different disease-associated isolates and analyze the genetic diversity.

In the second project we probe the interaction between natural *C. albicans* isolates and the host *in vivo* using the well-established model of oropharyngeal candidiasis (OPC). We investigate the broad spectrum of responses that are raised through natural variations in the pathogen. We highlight the requirement of the IL-17 pathway for preventing fungal outgrowth, irrespective of the natural diversity of *C. albicans*.

Finally, in the third project of this thesis, we aim to further investigate the correlation of a rapid and acute inflammatory response and infiltrating neutrophils and fungal clearance. We address this by co-infection experiments with a high-pathogenic isolate, which induces a rapid neutrophil response, and a low-pathogenic strain, for which we did not observe a significant neutrophil response.

Project II is accepted in Mucosal Immunology .

5 Project I

Low prevalence of *Candida albicans* in a local children population and comparable genetic diversity in isolates from healthy and diseased individuals

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5.1 Abstract

Knowledge on the prevalence of *Candida albicans* in healthy children is scarce. The aim of this work was to study the frequency of *Candida* spp. colonization in symptomless children and to investigate genetic diversity among isolates from healthy individuals and *Candida*-infected patients. The oral cavity, the skin or perianal region of 130 children were swabbed and the isolated *Candida* species were identified by plating on chromogenic fungal media and by MALDI-TOF analysis. Genetic diversity of isolates from healthy children and adults and diseased individuals was investigated by Multilocus Microsatellite typing. About 19.2 % of the children were colonized with *Candida* spp., mainly *C. albicans* (11.5%). Isolates from healthy children displayed a high genetic variation that is comparable

to the diversity found in healthy adults or in diseased populations. This study gives further insights on the epidemiology and distribution of *Candida* species in healthy children, and helps understanding the genotypic composition of *Candida* populations in diseased and healthy individuals.

5.2 Introduction

Skin and mucosal surfaces of the human body are colonized by a large variety of microorganisms, including several fungal species (Sanchez de Medina et al. 2013). *Candida* species are common components of the human microbiome that usually reside as harmless commensals in the mucosae of the oral, vaginal and gastrointestinal tract (Ghannoum et al. 2010). They pose a potential threat only if barrier integrity and host defenses are breached.

As an opportunistic pathogen *Candida* spp. may cause primary superficial infections of mucosal surfaces of the oral cavity or the vagina, but under specific circumstances it may also lead to invasive infections that are associated with a high mortality rate (Brown et al. 2012). In fact, *Candida* spp. are among the leading causes of nosocomial bloodstream infections (Wisplinghoff et al. 2014) and infection rates are rising also because of the increase in use of immunosuppressive medications or of invasive medical interventions and transplantations (Brown et al. 2012). Currently, 90% of all invasive fungal infections are caused by *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* (Mendes Giannini, M. J. S. et al. 2013; Delaloye, Calandra 2014).

C. albicans is the most prevalent fungus in the oral cavity (Ghannoum et al. 2010). In healthy adult individuals *Candida* colonization of the oral cavity is estimated to range between 17% to 75% (Cannon, Chaffin 1999; Javed et al. 2014), although numbers may vary according to age or geographical differences. Colonization occurs mainly by vertical transmission from the mother after birth but horizontal transmission is also frequent (Bliss et al. 2008). Infections usually arise from an endogenous source and are therefore associated with a prior colonization of other body sites (Pfaller 1996). In healthy children, numbers for *Candida* colonization range from 27% in the tonsillopharynx to 52% positive fecal samples (Gammelsrud et al. 2011).

Chronic mucocutaneous candidiasis (CMC), on the other hand, is a rare disease in which skin, nails and mucosal membranes are affected by *Candida* species (mainly *C. albicans*) (Puel et al. 2011). Primary immunodeficiencies have been found to be associated with CMC, leading to a defect in the Interleukin 17 and T-helper cells 17 mediated antifungal response or an impaired innate detection of fungal infections (Cypowyi et al. 2012; Milner, Holland 2013a).

Epidemiological studies concentrated so far mainly on the prevalence of *Candida* spp. in bloodstream infections (Wisplinghoff et al. 2014) or in individuals with different predisposition for oral candidiasis, such as HIV-infected or diabetes patients (Samaranayake 1992; Samaranayake et al. 2002; Martinez et al. 2013). Findings about the prevalence of commensal colonization in healthy children are rare and controversial (Martin, Wilkinson 1983; Akdeniz et al. 2002; Kadir et al. 2005; Smiline et al. 2012).

C. albicans is a diploid organism that shows a high genetic plasticity, even if the reproduction is largely clonal (Selmecki et al. 2010). Genetic changes such as mutations, loss of heterozygosity (LOH) and ploidy changes have been described (McManus, Coleman 2014; Bennett et al. 2014). Microevolution during infection (Bougnoux et al. 2006) and azole resistance (Coste et al. 2006) have been shown to be caused by LOH events. *C. albicans* was the first human fungal pathogen for which the full genomic sequence has been made available (Jones et al. 2004). The whole genome consists of 8 chromosome homologues and has a total length of 16 Mbp (Chibana 2000).

Due to the availability of the full sequence information, two PCR-based methods Multilocus sequence typing (MLST) and Multilocus microsatellite typing (MLMT) have been developed to examine the population structure and epidemiology of *C. albicans* and are considered the gold standard.

MLST is based on the sequence analysis of DNA fragments of up to 10 housekeeping genes and the detection of single nucleotide polymorphisms (Bougnoux et al. 2002). MLMT targets tandemly repeated sequences and assigns isolates to sequence types depending on the length of the fragment (Taylor, Fisher 2003).

Microsatellites are 1-6 bp long tandem nucleotide repeats, also called short tandem repeats (STR) and are distributed in the whole genome (Botterel et al. 2001). These short sequences are highly mutagenic. Repeated units undergo deletions or additions, resulting in variations in the number of copies. This occurs due to DNA polymerase slippage between repeated sequences (Jarne, Lagoda 1996; Lai, Sun 2003). MLMT targets tandemly repeated sequences by PCR amplification with primers flanking this regions (Taylor, Fisher 2003). The length of fragments at each locus can be related to an allelic profile and each profile corresponds to a Sequence Type (ST). Differences in length are caused by different numbers of repeats of the microsatellite (Botterel et al. 2001). Strains belonging to a specific ST are considered clonal and isolates can be assigned to clonal complexes based on allelic differences (Feil et al. 2004).

Population studies of *C. albicans* have shown that sub-groups can be differentiated by MLMT/MLST and isolates assigned to clades (Chavez-Galarza et al. 2010). Currently 18 major and minor clades/clonal complexes can be distinguished by MLST and some show phenotypic and geographical

accumulation (Odds et al. 2007). Several phenotypic attributes such as acid-phosphatase activity (MacCallum et al. 2009) and resistance to flucytosine and terbinafine (Odds 2009) have been correlated with specific clades by MLST, but no clear correlation between genotype lineage and virulence has so far been identified (MacCallum et al. 2009). A study on isolates originating from healthy volunteers, bloodstream and non-bloodstream infections using the microsatellite locus CEF3 showed no association between clonal complexes and virulence (Dalle et al. 2000). A subsequent study with the same isolates using eleven microsatellite loci indicated a distribution of the isolates within eight phylogenetic groups (L'ollivier et al. 2012).

So far, not much is known on the genotypic composition of the *C. albicans* populations colonizing the oral mucosae of healthy volunteers or patients suffering from mucocutaneous candidiasis or vulvovaginal candidiasis and candidemia patients. It is also unknown whether or not *C. albicans* strains colonizing patients with persistent or recurrent candidiasis are genotypically substantially different from those isolated from healthy controls. We were therefore aiming to assess the colonization rates by *Candida* spp. on the oral mucosae and skin of healthy children in southern part of Switzerland and to compare them by MLMT analysis with different disease-associated isolates.

5.3 Results

5.3.1 *Candida* spp. colonization frequency in healthy children is low

We investigated the colonization rates and distribution of *Candida* spp. in the oral cavity and skin of healthy children aged between 1 month and 16 years in Southern Switzerland (**Table 1**). Twenty-five out of 130 symptomless children (19.2%) were colonized by five *Candida* spp.. *C. albicans* was the most prevalent colonizer with 11.5 %. The second most frequent species was *Candida guilliermondii*, found in 3.8 % of all tested children. *Candida pelliculosa* and *C. parapsilosis* were recovered in 2 cases (1.5%) and *C. krusei* in one (0.8%). No individuals were colonized concurrently by two different species of *Candida*.

	Candida spp.	*C. albicans*	*C. guilliermondii*	*C. pelliculosa*	*C. parapsilosis*	*C. krusei*	total
n=	25	15	5	2	2	1	130
%	19.2	11.5	3.8	1.5	1.5	0.8	100

Table 1 Prevalence of *Candida* spp. in healthy children. Frequency of colonization by individual *Candida* spp. in 130 healthy children. Absolute numbers (n) and percentages (%) of *Candida* spp. distribution are indicated.

5.3.2 Healthy children are colonized by a clonal population

To investigate the diversity of the *C. albicans* populations in healthy children, we tested 12 isolates from healthy children by MLMT. To ensure a high discriminatory power, we analyzed three well described microsatellite markers *CDC3*, *EF3* and *HIS3* that are located on different chromosomes. *CDC3* is located downstream of the cell division cycle protein, *EF3* in the promoter region of the elongation factor 3, and *HIS3* downstream of the coding sequence of the imidazole glycerol phosphate dehydratase gene (Botterel et al. 2001). Fragment sizes of the reference strain SC5314 were consistent across independent measurements (data not shown) confirming the reproducibility of the method.

We examined 16 randomly selected individual colonies for each of 12 *C. albicans* isolates out of the population described above (**Table 2**) by MLMT. All three loci analyzed were identical in all colonies, indicating clonality of the *C. albicans* populations in the healthy children. On the other hand, the alleles varied greatly between the different *C. albicans* isolates (**Table 2**).

Isolate	N=	Bodysite	clonality (%)	*CDC3*		*EF3*		*HIS3*		ST
VP23	16	O	100	116	116	128	136	146	158	16
VP85	16	O	100	116	120	124	129	150	150	19
VP15	16	O	100	116	120	147	147	150	179	21
VP32	16	O	100	116	124	119	128	158	158	24
VP79	16	O	100	116	124	119	128	158	158	24
VP8	16	O	100	116	124	119	128	158	188	27
VP9	16	S	100	116	124	119	128	158	188	27
VP6	16	S	100	116	124	124	124	158	179	35
VP37	16	O	100	120	124	124	131	192	196	50
VP4	16	O	100	124	124	124	131	196	208	55
VP22	16	S	100	124	128	124	131	183	183	57
VP31	16	O	100	149	149	147	154	166	166	59

Table 2 Healthy children are colonized by a clonal population of *C. albicans*. 16 randomly picked colonies of the indicated isolates from the oral cavity (O) or skin (S) were examined by MLMT analysis to assess the clonality of the population. The fragment length of both alleles for the three loci *CDC3, EF3* and *HIS3* (in base pairs) and the corresponding STs are shown.

We further examined whether or not the same clonal *C. albicans* would be present in all different anatomical sites of the same individual. We performed MLMT on *C. albicans* isolates that were collected from different body sites including oral cavity, skin, and perianal region of a child affected with oral candidiasis (002) and a symptomless child (101). Clonality was also observed across different collection sites within an individual (**Table 3**). Moreover, *C. albicans* populations were stable at these different body sites over the investigated time of three weeks (**Table 3**). .Because these results confirmed clonality of *C. albicans* at the level of an individual, we used only one colony of each individual in the consecutive experiments/analysis. Among the 14 isolates from healthy children (**Table**

2+3), we identified 12 different STs. Only one isolate (101, ST 54) from the population of healthy children had a homologous allelic profile at all three loci.

Isolate	N=	Origin	Bodysite	timepoint	clonality (%)	*CDC3*	*EF3*	*HIS3*	ST
002	15	MC	O	week 0	100	120 120	128 128	146 146	49
002	15		P	week 0	100	120 120	128 128	146 146	
002	15		S	week0	100	120 120	128 128	146 146	
002	15		P	week 1	100	120 120	128 128	146 146	
101	15	V	O	week 0	100	124 124	124 124	188 188	54
101	15		P	week 0	100	124 124	124 124	188 188	
101	15		O	week 1	100	124 124	124 124	188 188	
101	15		P	week 1	100	124 124	124 124	188 188	
101	15		0	week 2	100	124 124	124 124	188 188	
101	15		P	week 2	100	124 124	124 124	188 188	
101	15		0	week 3	100	124 124	124 124	188 188	
101	15		P	week 3	100	124 124	124 124	188 188	

Table 3 Clonality of *C. albicans* isolates across different body sites. The isolates 101 (from a healthy children) and 002 (from oral candidiasis patient) were collected from the skin (S), the oral cavity (O), perianally (P), and 15 randomly selected individual colonies of each isolate were examined by MLMT. The fragment length of both alleles for the three loci *CDC3, EF3* and *HIS3* (in base pairs) and the corresponding STs are shown.

5.3.3 *C. albicans* isolates show genetic variation

After assessing clonality of the *C. albicans* population within individuals, we were interested in the diversity among individuals. When examining the fragment lengths of the *CDC3*, *EF3* and *HIS3* microsatellites in the collection of isolates from healthy children (**Table 2**), it became obvious that the isolates displayed greatly variable alleles.

We then compared the isolates from the healthy children cohort to those from healthy adults (**Table 4).** One colony of each individual was tested, since we showed colonization with a clonal population before (**Table 2Table 3**). Similar to isolates from healthy children, we detected a high diversity in fragment length of the alleles from the three tested loci.

The alleles were mostly heterologous at the analyzed loci, with three isolates (V196, V67, VolG, ST 47) being homologous at all loci, compatible with our findings in isolates from healthy children. No increased frequency of any particular allele or resulting ST was found. Allele size at each of the locus defined the ST. Isolates showing the same allele size at all loci were assigned to one ST. Therefore, the high genetic variation and the high number of corresponding STs was independent of the age of the population.

Isolate	*CDC3*		*EF3*		*HIS3*		ST
V201	112	112	123	128	146	158	1
V202	112	112	123	128	146	158	1
V162	112	116	122	128	158	158	4
V44	112	116	123	128	146	158	6
V128	116	116	119	128	158	158	9
V184	116	120	123	128	146	162	18
V66big	116	120	123	128	146	162	18
V18	116	120	125	128	150	150	20
V91	116	120	125	128	150	150	20
V107	116	124	119	119	158	158	22
V53	116	124	119	119	158	158	22
V73	116	124	119	119	158	158	22
V79	116	124	119	119	158	158	22
V93	116	124	119	119	162	162	23
V197	116	124	119	128	158	158	24
V199	116	124	119	128	158	158	24
V203	116	124	119	128	158	158	24
V55	116	124	119	128	200	200	29
V68	116	124	123	128	146	150	33
V11	116	124	124	123	178	184	34
V48	116	124	124	124	178	182	36
Vc4-1	116	124	128	128	158	212	39
V105	116	128	119	128	158	158	41
V164	116	128	119	128	158	158	41
V27	116	128	119	128	158	158	41
V28	116	128	126	126	162	178	42
V204	116	128	128	137	150	150	43
V205	116	128	128	137	150	150	43
V71	116	128	128	137	150	150	43
V196	120	120	122	122	146	146	47
V67	120	120	122	122	146	146	47
VolG	120	120	122	122	146	146	47
V86	120	124	128	128	146	146	51
V24	124	124	123	134	182	204	53

Table 4 Genetic diversity of *C. albicans* isolates from healthy adults. MLMT analysis of 34 isolates from healthy individuals. The fragment length of both alleles for the three loci *CDC3, EF3* and *HIS3* (in base pairs) and the corresponding STs are shown.

We expanded our analysis to isolates from candidiasis patients, including oral candidiasis and chronic mucocutaneous candidiasis (grouped together as mucocutaneous candidiasis), vulvovaginal candidiasis and candidemia (**Table 5**). Similarly to isolates from healthy individuals, *C. albicans* isolates from different patient groups displayed a broad diversity in fragment sizes of the alleles at the three loci analyzed. In agreement with results from healthy adults and children there was no increased frequency of a specific allelic combination or the resulting ST. Heterologous loci were detected in the majority of the isolates and in some isolates both alleles gave rise to PCR products of the same length. Three STs (ST 47, ST 54, ST 14) in total, consisted of homologous allelic combinations in all three loci. In conclusion, genetic variation in the tested loci *CDC3*, *EF3* and *HIS3* is independent of the origin and the age of the tested population.

Isolate	Origin	*CDC3*		*EF3*		*HIS3*		ST
065 I M1	MC	112	112	123	128	146	162	2
CMo2	MC	112	112	128	128	146	158	3
CMo3	MC	112	112	128	128	146	158	3
CMo4	MC	112	112	128	128	146	158	3
Cla	MC	116	116	119	128	174	183	10
529L	MC	116	116	128	131	166	166	15
062 I M1	MC	116	116	128	137	150	150	17
005 I P1	MC	116	124	119	128	158	183	26
006 I P1	MC	116	124	119	131	166	198	31
004 I P1	MC	116	128	119	128	146	158	40
Cag	MC	124	124	124	132	188	200	56
ICM70	VVC	112	116	128	128	158	158	7
ICM225	VVC	116	116	122	129	258	258	12
ICM24	VVC	116	116	128	128	158	158	14
ICM555	VVC	116	116	128	137	150	150	17
VVG5	VVC	116	120	125	128	150	150	20
ICM550	VVC	116	124	119	119	158	158	22
ICM586	VVC	116	124	119	119	158	158	22
ICM231	VVC	116	124	119	128	158	158	24
ICM55	VVC	116	124	119	128	158	158	24
ICM606	VVC	116	124	119	128	158	158	24
ICM23	VVC	116	124	119	128	192	192	28
ICM551	VVC	116	124	123	123	178	182	32
ICM230	VVC	116	124	128	128	158	158	37
VVG2	VVC	116	128	128	137	150	154	44
ICM557	VVC	116	128	128	139	150	150	45
VVG4	VVC	116	146	128	128	158	162	46
ICM552	VVC	120	120	122	128	150	150	48
ICM243	VVC	128	128	128	137	150	158	58
UC820	INV	112	116	123	128	146	146	5
ICM36	INV	112	136	137	137	150	158	8
ICM4	INV	116	116	122	128	150	150	11
ICM6	INV	116	116	125	136	150	150	13
ICM1	INV	116	116	125	136	150	150	13
ICM104	INV	116	124	119	128	158	158	24
SC5314	INV	116	124	119	128	158	158	24
ICM3	INV	116	124	119	128	158	166	25
ICM7	INV	116	124	119	128	234	246	30
ATCC14053	INV	116	124	128	128	158	179	38
ICM5	INV	124	124	119	128	158	158	52

Table 5 Genetic diversity *of C. albicans* isolates from diseased individuals. MLMT analysis of isolates from oral candidiasis and CMC patients (MC), vaginal isolates form vulvovaginal patients (VVC) and blood isolates from candidemia patients (INV). The fragment length of both alleles for the three loci *CDC3, EF3* and *HIS3* (in base pairs) and the corresponding STs are shown.

Given the large diversity in length of microsatellites alleles identified, we aimed at analyzing the distribution of the different alleles and therefore monitored the frequency of each of the allelic combinations for the three loci analyzed. Various combinations of these alleles resulted in 14, 25, and 32 different allele combinations for *CDC3, EF3* and *HIS3* locus, respectively (**Figure 3**). Amplification of *EF3* and *HIS3* led to the highest variability in fragment size lengths, resulting in a discriminatory power of 0.89 and 0.88, respectively. *CDC3* alone just led to a discriminatory power of 0.82, in other words a 82% probability to separate two unrelated strains (Hunter, Gaston 1988). Most of the allelic

combinations were heterologous, but some frequently found allelic pairs appeared also to be homologous. At the *CDC3* locus we detected 6 homologous allelic combinations, 8 combinations appeared homologous at *EF3* and 10 were homologous at the HIS3 locus. The frequency of different allele pairs varied greatly at each locus, with some shared among many isolates and others occurring only rarely (**Figure 3**).For the *CDC3* locus, the 116:124 allele pair was by far the most frequent one found in 33 isolates. The most frequent allele pairs for *EF3* and *HIS3* were the allelic combinations 119:128 with 24 isolates and 158:158 with 25 isolates, respectively.

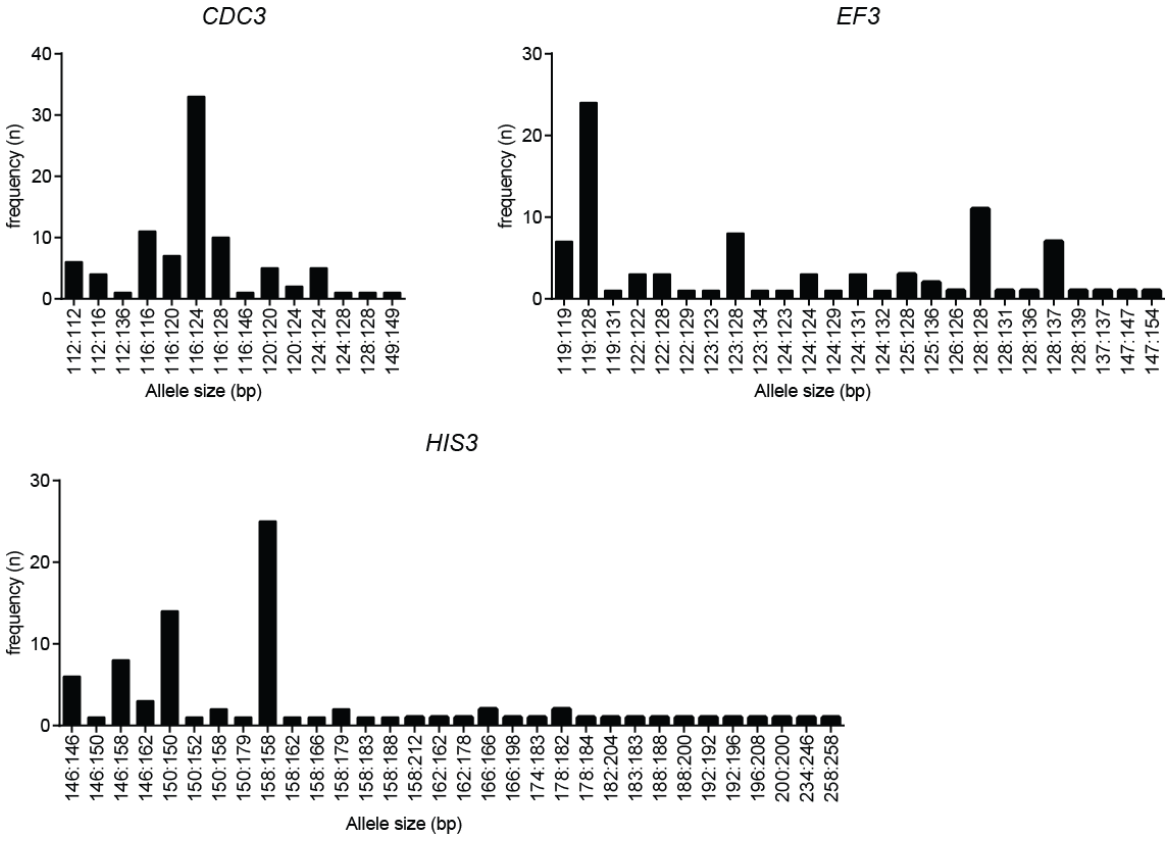

Figure 3 Variation of allelic relations in all three loci. Isolates from healthy individuals and candida-affected patients with symptoms tested by MLMT analysis with *CDC3, EF3, HIS3* locus.

Based on these allelic profiles, we assigned distinct STs (**Table 2-Table** 5**,** column 'ST'). Isolates sharing a ST were defined as clonal. When assessing all 88 isolates, including those from healthy children (**Table 2Table** 3) and adults (**Table 4**) and from diseased individuals (**Table 5**), 59 STs could be defined in total (**Figure 4**). Each allelic combination from all three tested loci were assigned to one ST. This led to a discriminatory power of 0.979, which describes an approx. 98% probability to distinguish two unrelated STs (Hunter, Gaston 1988). The STs showed very different abundances, with ST 24 being the most frequent one. The majority (47 out of 59 STs) are less abundant and contain only one isolate.

All isolates from oral candidiasis patients, except 529L, were from individuals living in the southern part of Switzerland. They all belong to different STs, supporting the hypothesis that there is no clonal regional population. This is also shown in Table 2 and 4 for isolates from healthy individuals, all isolated from the same geographical area (southern Switzerland). Isolates from the adult healthy population showed an accumulation with at least three isolates in STs 22, 24, 41, 43 and 47. The 18 isolates from vulvovaginal candidiasis patients formed 15 STs. Two isolates clustered in ST 22 and 24 but also isolates from other origins belong to these STs. ST 24 was the most common one involving 10 isolates from several distinct origins. The three mucocutaneous candidiasis isolates (Table S1) sharing ST 3 belong to one family, indicating that all tested family members carry the same *C. albicans* isolate.

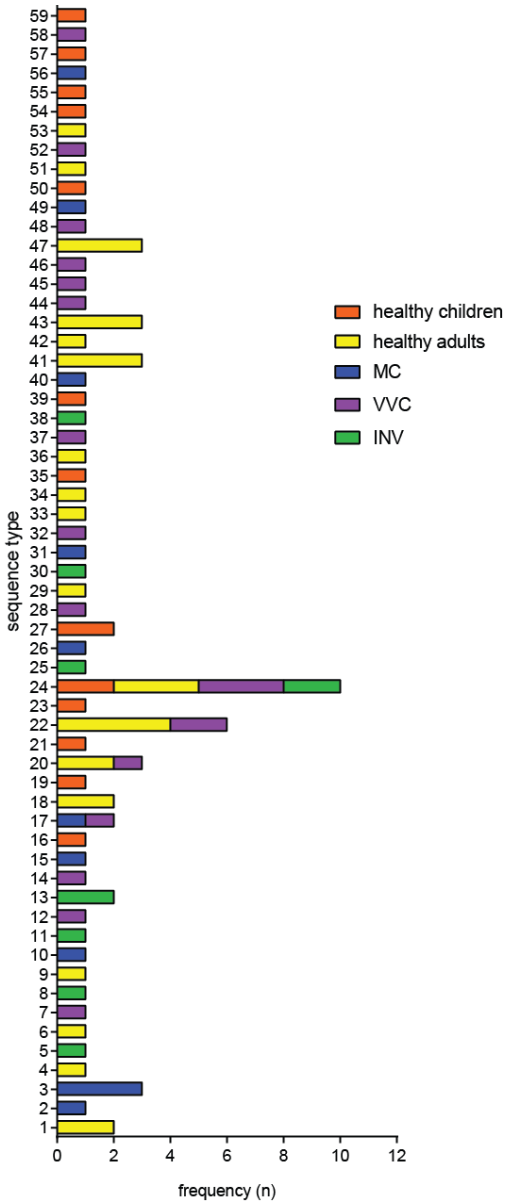

Figure 4 Distribution of *C. albicans* isolates in STs. Frequency of isolates from healthy children, healthy adults or diseased individuals separated in vulvovaginal candidiasis (VVC), invasive candidiasis (INV) and mucocutaneous candidiasis (MC) patients in the different sequence types.

5.3.4 Comparable distribution of commensal and disease-associated isolates in clonal complexes

After dividing the 88 isolates in 59 STs clonal complexes were assigned of STs with only one divergent allele from all three loci. The allelic profiles obtained through the MLMT analysis could be clustered into clonal complexes by an algorithm that is based upon related STs (BURST (Aanensen, Spratt 2005).

Applying this algorithm to our 59 STs led to the formation of four clonal complexes (**Figure 5**). Combination of STs within a clonal complex indicates their hypothetical phylogenetic relationship (Feil et al. 2004; Spratt et al. 2004). The most abundant ST 24 was part of the largest clonal cluster 1 comprising 32 isolates grouped in 15 different ST. ST 24 acted as the primary founder of the complex, which also comprised a subcomplex of six ST with ST 37 acting as a subgroup founder. Clonal complexes 2 and 3 consisted of five and four STs, respectively. Clonal complexes 4 comprised only two STs. 33 STs could not be assigned to any clonal complex (singletons). The large number of singletons underlined the high degree of diversity among the isolates tested.

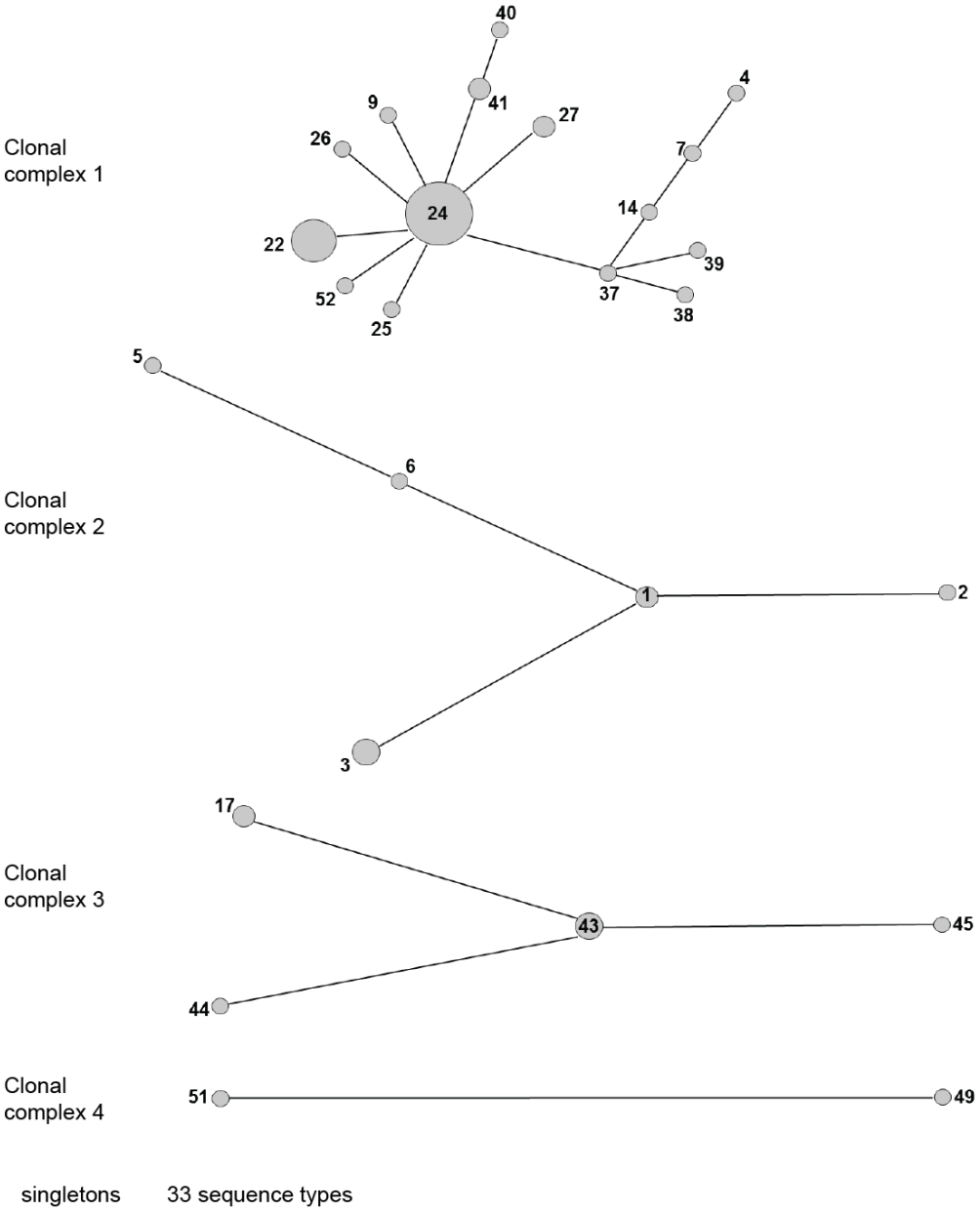

Figure 5 Clustering of the STs in four clonal complexes by BURST analysis. Four clonal complexes of variable size and complexity are shown. The size of the circle denotes the number of isolates within each ST.

The distribution of isolates within clonal complexes according to their origin was mixed. In clonal complex 1, 17 of all isolates were from healthy and 15 from diseased individuals, respectively (**Figure 6**). In addition, the smaller clonal complexes 2, 3 and 4 comprised isolates from healthy and diseased individuals in almost equal proportions. Also the singletons were isolates from both groups (23 from healthy individuals and 16 from diseased individuals resulting in 33 ST. The isolates from healthy children were restricted to clonal complex 1 or they were singletons. This result, however, might have been a consequence of the limited number of isolates in the group of healthy children.

We could not detect an accumulation of any isolates from a specific population in STs or clonal complexes but rather a high variation within isolates.

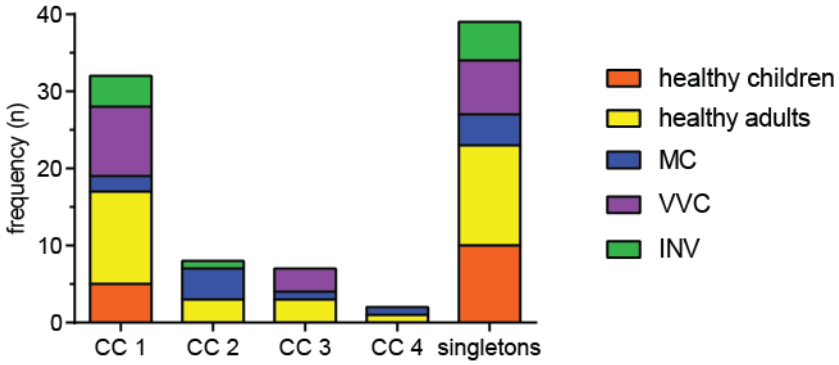

Figure 6. Isolates from healthy and symptomatic individuals segregated to similar proportions into clonal complexes. Distribution of isolates from healthy individuals (children and adults) and diseased-individuals in clonal complexes (CC) and singletons.

5.4 Discussion

We observed a low frequency of *Candida* colonization in healthy children but we detected a high genotypic variation among isolates from different individuals. MLMT analysis confirmed that the findings in the healthy children population apply also to adults and diseased individuals affected with mucocutaneous, vulvovaginal or invasive candidiasis and indicated a high genetic variation among isolates.

Candida colonization is common in healthy individuals. In the present study, we re-evaluated the prevalence of *Candida spp*. colonization in healthy children from a restricted and defined geographical area. Surprisingly, we found that only every fifth child was colonized. *C. albicans*, the most prevalent species, was detected in one out of nine children. In the literature, the reported overall prevalence of *Candida* spp. in children with a similar age ranges between 26 % in 0-12 year old children in Turkey and 71% in 10-year-old children in the United Kingdom (Martin, Wilkinson 1983; Kadir et al. 2005). Based on our data, these numbers appear to be overestimates, although colonization rates may also be influenced by age, population and dietary intake (Kadir et al. 2005). Differences may also exist due to the geographic location and investigated body site.

C. guilliermondii, *C. pelliculosa*, *C. parapsilosis* and *C. krusei* were also isolated, albeit at rather low frequencies. This correlates well with previous reports on the distribution of *Candida* spp. and their clinical importance (Gammelsrud et al. 2011; Kadir et al. 2005). *C. pelliculosa* (teleomorph *Pichia anomala*) has only been occasionally reported in cases of nosocomial candidemia (Paula et al. 2006).

This yeast is usually isolated from the environment, including vegetables and soils (Kalkanci et al. 2010). We could not detect *C. glabrata* or *C. tropicalis* in the tested population, even though *C. glabrata* is the second or third most common *Candida* species in superficial and invasive infections due to the natural resistance to antimycotic therapy (Fidel, JR et al. 1999). In agreement with previous studies, we showed that prevalence of *Candida* spp. colonization is dependent on the geographical area(Singhi et al. 2008). This study strengthened the need for local epidemiology studies.

Nowadays two methods established to be the gold standard for genetic population studies, MLMT and MLST. Analysis of an organism's genetic diversity requires a method that gives reliable and reproducible results. We used MLMT analysis with the *CDC3*, *EF3* and *HIS3* loci.

A disadvantage of MLMT is the lack of a public database such as the one available for MLST (Bougnoux et al. 2004). Fragment sizes obtained by MLMT can differ among laboratories depending on the electrophoresis method, instrument and fluorescent labels used, by the distance of the fragment from the used standard and the analysis software. Both methods have a similar discriminatory power and results of assigned groups obtained by the two methods correlate well (Garcia-Hermoso et al. 2007; L'ollivier et al. 2012). Microsatellites are robust markers, faster and cost-effective due to a higher throughput compared to MLST (Virk et al. 1999).We used MLMT analysis in a multiplex option with three loci, therefore the method is faster and cost-effective due to a higher throughput compared to MLST (Virk et al. 1999).

We observed clonal populations within, but no clonality among healthy individuals. This is in agreement with results from other studies in which one individual was shown to be stably colonized by a single strain of *C. albicans* in several body sites over time (Mendes Giannini, M. J. S. et al. 2013; Hattori et al. 2006; Shimizu et al. 2011). Other studies applying DNA fingerprinting and randomly amplified polymorphic DNA analysis on samples from the oral cavity, vaginal mucosa and rectum from woman with candidiasis (Xu et al. 1999) or MLST analyses with isolates from CMC patients (Moorhouse et al. 2016) showed similar results.

This study is limited to isolates collected at a single time point and over a short time period of three weeks and lacks the inclusion of long-term follow-up samples. This would allow the detection of microvariations, defined by small genetic rearrangements (Dalle et al. 2008). Isolate 101 appeared to be stable at all microsatellite loci over three weeks. We showed clonality in all colonized bodysites and over time, supporting the argument of a clonal mode of propagation (Gräser et al. 1996; L'ollivier et al. 2012). We therefore assumed that the use of one colony per individual is representative for the colonizing *Candida* population (Dalle et al. 2008). The Patient colonized with isolate 002 could only be

investigated for one week, due to antimycotic treatment of the patient and the potential clearance of the fungus.

The 88 isolates tested in this study clustered in 59 STs based on differences in fragment size. Differences in length of the microsatellites arise through differences in the number of tandem repeats. Mutation rates for Microsatellites vary between 10^{-4} and $5x10^{-6}$ (Bretagne et al. 1997). Several isolates were homologous at one locus and three STs consisted of homologous allelic combinations in all three tested loci. This could be the result of same length of the amplified fragments by convergent evolutionary events or the isolates were haploid at this locus through loss of heterozygosity (Dalle et al. 2008).

Mutational changes of microsatellites mostly increase or decrease the repeat number by one; this occurs through a mechanism called replication slippage, however motif composition can also undergo mutation (Jarne, Lagoda 1996). The latter can only be detected by sequencing, which was not carried out in this study.

Almost each individual harbored one isolate of a specific ST. Isolates belonging to the same ST may be identical due to relatedness of the colonized individuals. Isolates from ST 3 were all isolated from one family affected by CMC. This confirms the results from a study that showed intrafamilial transmission of *Candida* in families affected with Crohn`s disease (Bougnoux et al. 2006). Other STs might be further separated by the use of more discriminate markers than used in this study.

In the present study we could separate two unrelated isolates with a probability of 98%. The comparison of isolates from healthy children versus those from healthy adults did not reveal a distinct pattern, neither did the comparison of healthy- versus disease-associated isolates. This indicated that genotypically characterized ST or clonal complexes do not correlate with the health status and the demographics of the individuals from whom they are isolated.

STs were grouped in clonal complexes by their similarity to an allelic profile from a founder ST. Four clonal complexes were determined. Clonal complex 1, with the founder ST 24, was the largest of all complexes deduced from 32 isolates in 15 distinct STs and comprised a subgroup with the ST 37 as a founder. In all clonal complexes, isolates from healthy and diseased individuals were almost equally frequent. The group of singletons (33 isolates) consisted of a larger proportion of isolates from healthy individuals, but no clonal complex consisted exclusively of isolates from a specific group.

The STs formed independently from the anatomical site. We could not distinguish the isolates by their anatomical site of isolation, but a previous study has shown that the site of isolation is not associated

with a specific clade/clonal complex (Odds et al. 2007). But MLST analysis showed an association between geographical origin and organ distribution of oral, vaginal and blood isolates amongst the major clades within western European isolates (Odds et al. 2007). Overrepresented allelic combinations for all three loci proved, in agreement with a previous study, the equal distribution of all tested isolates (L'ollivier et al. 2012). It was proposed that some allelic combinations of the *HIS3* locus are associated with a pathogenic profile (L'ollivier et al. 2012). We could not detect distinct alleles and a correlation with the origin of the isolates.

The number of patients who develop fungal infections, as candidiasis and candidemia, has increased dramatically in the last decades, particularly in immunosuppressed patients. A more detailed knowledge of the epidemiology of *Candida* species in children will be helpful to predict infection patterns, through the knowledge of possible vertical and horizontal transmissions, and eventually to develop effective prevention or/and treatment schedules. In the present study we showed a low prevalence of *C. albicans* in healthy children, probably by the dependence on the geographical area. Furthermore, we could proof extensive genetic diversity among isolates from healthy individuals and patients.

5.5 Material and Methods

5.5.1 Study design for the collection of *Candida* spp. isolates from healthy children and oral candidiasis patients

Samples from 130 healthy children and 6 patients with oral candidiasis were collected in the frame of a clinical trial in Ticino, Switzerland. The cantonal ethics committee of the Canton of Ticino, Switzerland approved the study (approval number CE 2669).

The study population consisted of children between 1 month and 16 years. During a routine check visit of their child, parents were asked to participate in the study and to give their consent to the participation of their child in the study. This control visit coincided with the Screening visit. Demographics, medical history and concomitant therapies were recorded. Immunosuppression, immune disease and antibiotic treatment were used as exclusion criteria. A complete physical examination was carried out and swabs from mouth region/throat, skin and perianal region were taken by the treating physician or a study nurse. Follow up swabs were taken at home by the parents at weekly intervals (1 swab/week) for three weeks, after careful instruction of the parents on how to take

the swabs, which were then sent by mail to the laboratory. Paediatric patients with a diagnosis of oral candidiasis were immediately treated after the first swab.

5.5.2 *Candida albicans* isolates used for MLMT analysis

All 88 *C. albicans* isolates used in this study are listed in Supplementary Table 1. They comprise 14 isolates from healthy children, 34 from healthy adults and 40 from patients with oral candidiasis or chronic mucocutaneous candidiasis, vulvovaginal candidiasis or candidemia. All isolates were grown on Sabouraud agar (Biomérieux, Switzerland) at 37°C for one day. For long term storage, they were kept in YPD medium supplemented with 20% glycerol at -80°C.

5.5.3 Species identification

For *Candida* species identification the isolates were grown on chromogenic Candida agar plates (Can2; Biomérieux, Switzerland) for a preliminary morphological identification. They were subsequently inoculated on Sabouraud Agar and identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) as described by (Benagli et al. 2012). Small amounts of *Candida* cells were transferred from the Sabouraud agar plate directly on a 48-position stainless steel FlexiMass™ target plate (Shimadzu Biotech, Kyoto, Japan) and overlaid with 1 µl of α-cyano-4-hydroxycinnamic acid (CHCA)-Matrix solution containing 40 mg/ml CHCA in acetonitrile/ethanol/water (1:1:1) supplemented with 3% trifluoroacetic acid. All mass spectra were acquired using an AXIMA Confidence™ (Shimadzu Biotech, Kyoto, Japan) mass spectrometer. The peak list with m/z values were compared to reference SuperSpectra™ of *Candida* spp. present in the SARAMIS™ (Spectral Archive And Microbial Identification System) database to produce identifications at the species level.

5.5.4 Multilocus Microsatellite typing (MLMT)

DNA was extracted from *C. albicans* colonies grown on Sabouraud agar using InstaGene matrix (BioRad) following the manufacturer's instructions. The Microsatellites used were *CDC3*, *EF3; HIS3* (Table 1) (Bretagne et al. 1997; Botterel et al. 2001; Sampaio et al. 2005). One primer of each set was 5' labelled with hexachloro-fluorescein (for CDC3, HIS3) or fluorescein amidite (for EF3). PCR reactions were carried out according to standard protocols using HotStarTaq Polymerase (Qiagen) and a 2720 Thermal Cycler (Applied Biosystems). Gene products were analyzed on a 3500 Genetic Analyzer (Applied Biosystems) using a GeneScan 600 Liz dye size standard (Applied Biosystems) and the GeneMapper v4.1 software (Applied Biosystems). To assess reproducibility, the bloodstream isolate SC5314 (Gillum et al. 1984b) was used as a reference strain in all fragment analyses to prove consistency of electrophoresis and DNA extraction. Molecular data were analyzed by eBurst ver. 3 (http://eburst.mlst.net/) (Aanensen, Spratt 2005). The use of the combination of 3 markers leads to a

discriminatory power of 0.98, as calculated by the Simpson index of diversity (Hunter, Gaston 1988). A high discriminatory power ((close to 1) indicates that unrelated isolates can be well distinguished from the rest of the population (Hunter 1990).

Micro-satellite	repeat motif	primer sequence (5'–3')	fragment size (bp)	locus	Gene ID
CDC3	(AGTA)n	fwd: CAGATGATTTTTTGTATGAGAAGAA rev: CAGTCACAAGATTAAAATGTTCAAG	from 112 to 149	*CDC3*	3644638
EF3	(TTTC)n (TTC)n	fwd: TTTCCTCTTCCTTTCATATAGAA rev: GGATTCACTAGCAGCAGACA	from 112 to 154	*CEF3*	3647040
HIS3	(ATTT)n	fwd: TGGCAAAAATGATATTCCAA rev: TACACTATGCCCCAAACACA	from 146 to 258	*HIS3*	3647777

Table 6. Microsatellites used in this study. The repeat motif, primer sequences, the obtained fragment sizes, locus and Gene ID from NCBI GenBank are indicated.

5.6 Supplementary files

Isolate	Origin	bodysite	source	ST
V201	V	VS	Clinical laboratory of the Canton Ticino Hospital	1
V202	V	VS	Clinical laboratory of the Canton Ticino Hospital	1
065 I M1	MC-OC	O	clinical trial CE2669	2
CMo2	MC-CMC	O	kindly obtained from B. Moshe-Shoshan	3
CMo3	MC-CMC	P	kindly obtained from B. Moshe-Shoshan	3
CMo4	MC-CMC	O	kindly obtained from B. Moshe-Shoshan	3
V162	V	VS	Clinical laboratory of the Canton Ticino Hospital	4
UC820	INV	BC	ATCC MYA-3573 (Lehrer, Cline 1969)	5
V44	V	O	Clinical laboratory of the Canton Ticino Hospital	6
ICM70	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	7
ICM36	INV	BC	Clinical laboratory of the Canton Ticino Hospital	8
V128	V	VS	Clinical laboratory of the Canton Ticino Hospital	9
Cla	MC-CMC	O	kindly obtained from F. Angelini	10
ICM4	INV	BC	Clinical laboratory of the Canton Ticino Hospital	11
ICM225	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	12
ICM6	INV	BC	Clinical laboratory of the Canton Ticino Hospital	13
ICM1	INV	BC	Clinical laboratory of the Canton Ticino Hospital	13
ICM24	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	14
529L	MC-OC	O	kindly obtained from J. Naglik (Rahman et al. 2007)	15
VP23	V	O	clinical trial CE2669	16
062 I M1	MC-OC	O	clinical trial CE2669	17
ICM555	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	17
V184	V	VS	Clinical laboratory of the Canton Ticino Hospital	18
V66big	V	O	Clinical laboratory of the Canton Ticino Hospital	18
VP85	V	O	clinical trial CE2669	19
V18	V	VS	Clinical laboratory of the Canton Ticino Hospital	20
V91	V	O	Clinical laboratory of the Canton Ticino Hospital	20
VVG5	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	20
VP15	V	O	clinical trial CE2669	21
V107	V	O	Clinical laboratory of the Canton Ticino Hospital	22
V53	V	VS	Clinical laboratory of the Canton Ticino Hospital	22
V73	V	O	Clinical laboratory of the Canton Ticino Hospital	22
V79	V	VS	Clinical laboratory of the Canton Ticino Hospital	22
ICM550	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	22
ICM586	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	22
V93	V	O	Clinical laboratory of the Canton Ticino Hospital	23
V197	V	VS	Clinical laboratory of the Canton Ticino Hospital	24
V199	V	VS	Clinical laboratory of the Canton Ticino Hospital	24
V203	V	VS	Clinical laboratory of the Canton Ticino Hospital	24
VP32	V	O	clinical trial CE2669	24
VP79	V	O	clinical trial CE2669	24
ICM231	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	24
ICM55	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	24
ICM606	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	24
ICM104	INV	BC	Clinical laboratory of the Canton Ticino Hospital	24
SC5314	INV	BC	(Gillum et al. 1984)	24
ICM3	INV	BC	Clinical laboratory of the Canton Ticino Hospital	25
005 I P1	MC-OC	P	clinical trial CE2669	26
VP8	V	O	clinical trial CE2669	27

VP9	V	S	clinical trial CE2669	27
ICM23	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	28
V55	V	VS	Clinical laboratory of the Canton Ticino Hospital	29
ICM7	INV	BC	Clinical laboratory of the Canton Ticino Hospital	30
006 I P1	MC-OC	P	clinical trial CE2669	31
ICM551	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	32
V68	V	O	Clinical laboratory of the Canton Ticino Hospital	33
V11	V	O	Clinical laboratory of the Canton Ticino Hospital	34
VP6	V	S	clinical trial CE2669	35
V48	V	O	Clinical laboratory of the Canton Ticino Hospital	36
ICM230	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	37
ATCC14053	INV	BC	(Skowronski, Feldman 1989)	38
Vc4-1	V	O	Clinical laboratory of the Canton Ticino Hospital	39
004 I P1	MC-OC	P	clinical trial CE2669	40
V105	V	O	Clinical laboratory of the Canton Ticino Hospital	41
V164	V	VS	Clinical laboratory of the Canton Ticino Hospital	41
V27	V	O	Clinical laboratory of the Canton Ticino Hospital	41
V28	V	VS	Clinical laboratory of the Canton Ticino Hospital	42
V204	V	VS	Clinical laboratory of the Canton Ticino Hospital	43
V205	V	N/A	Clinical laboratory of the Canton Ticino Hospital	43
V71	V	O	Clinical laboratory of the Canton Ticino Hospital	43
VVG2	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	44
ICM557	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	45
VVG4	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	46
V196	V	VS	Clinical laboratory of the Canton Ticino Hospital	47
V67	V	VS	Clinical laboratory of the Canton Ticino Hospital	47
VolG	V	VS	Clinical laboratory of the Canton Ticino Hospital	47
ICM552	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	48
002	MC-OC	O, S, P	clinical trial CE2669	49
VP37	V	O	clinical trial CE2669	50
V86	V	O	Clinical laboratory of the Canton Ticino Hospital	51
ICM5	INV	BC	Clinical laboratory of the Canton Ticino Hospital	52
V24	V	O	Clinical laboratory of the Canton Ticino Hospital	53
101	V	O,P	clinical trial CE2669 (Schönherr et al.)	54
VP4	V	O	clinical trial CE2669	55
Cag	MC-CMC	O	kindly obtained from D. Firinu (Schönherr et al.)	56
VP22	V	S	clinical trial CE2669	57
ICM243	VVC	VS	Clinical laboratory of the Canton Ticino Hospital	58
VP31	V	O	clinical trial CE2669	59

Supplementary Table 1. All isolates used in this study. Isolates clustered by origin in volunteers (V), oral candidiasis (MC-OC), chronic mucocutaneous candidiasis (MC-CMC), vulvovaginal candidiasis (VVC) and candidemia (INV). The bodysite of isolation or kind of sample is indicated with oral cavity (O), skin (S), perianal region (P), vaginal swab (VS) and blood culture (BC). The source and ST (ST) are shown.

6 Project II

The intraspecies diversity of *C. albicans* triggers qualitatively and temporally distinct host responses that determine the balance between commensalism and pathogenicity

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F.A.S. and S.L.L. designed the study and wrote the paper. F.A.S, E.G., A.G., N.S., F.K., F.S., and K.T.W. performed experiments and analyzed data. U.H. performed transmission electron microscopy. G.T.L. and N.P. performed experiments on fungal cell wall composition and β-glucan exposure. M.E.B and C.D. provided fungal strains and provided advice. C.F.C. and S.L.L. supervised the project.

Running Head: Diversity of *C. albicans* pathogenicity *in vivo*

6.1 Abstract

The host immune status is critical for preventing opportunistic infections with *C. albicans*. Whether the natural fungal diversity that exists between *C. albicans* isolates also influences disease development remains unclear. Here, we used an experimental model of oral infection to probe the host response to diverse *C. albicans* isolates *in vivo* and found dramatic differences in their ability to persist in the oral mucosa, which inversely correlated with the degree and kinetics of immune activation in the host. Strikingly, the requirement of IL-17 signaling for fungal control was conserved between isolates, including isolates with delayed induction of IL-17. This underscores the relevance of IL-17 immunity in mucosal defense against *C. albicans*. In contrast, the accumulation of neutrophils and induction of inflammation in the infected tissue was strictly strain-dependent. The dichotomy of the inflammatory neutrophil response was linked to the capacity of fungal strains to cause cellular damage and release of alarmins from the epithelium. The epithelium thus translates differences in the fungus into qualitatively distinct host responses. Together, this study provides a comprehensive understanding of the antifungal response in the oral mucosa and demonstrates the relevance of evaluating intraspecies differences for the outcome of fungal-host interactions *in vivo*.

6.2 Introduction

Candida albicans is an important pathobiont in humans that can cause diseases ranging from mild superficial symptoms affecting the skin and the mucosa to severe systemic infections associated with high mortality rates. The development of disease is generally attributed to defects in host resistance. Individuals with impaired cellular immunity such as AIDS patients with low $CD4^+$ T cell counts or glucocorticoid-treated patients display an increased susceptibility to fungal infection and frequently display symptoms of oral thrush (Kirkpatrick 2001). More recently, primary immunodeficiencies affecting the T cell compartment and in particular the Th17 subset of T cells, which are associated with chronic forms of mucocutaneous candidiasis (CMC), revealed a critical role of the IL-17 pathway, in particular the two IL-17 family members IL-17A and IL-17F, in host protection from *C. albicans* (Puel et al. 2012).

The discovery of genetic defects associated with fungal infections and experimental work with mouse infection models helped to decipher the mechanisms of antifungal defense in the mucosal epithelium. IL-17A and IL-17F are rapidly induced in response to *C. albicans* by different tissue-specific innate cellular sources (Gladiator et al. 2013; Conti et al. 2014; Kashem et al. 2015a). They act in a synergistic manner (Gladiator et al. 2013; Whibley et al. 2016) and target the epithelium for induction of antimicrobial peptides (Conti et al. 2016; Trautwein-Weidner et al. 2015b). The IL-17-dependent epithelial response is complemented by neutrophils, which rapidly infiltrate the infected tissue in an IL-17-independent manner (Trautwein-Weidner et al. 2015b) and prevent fungal growth and dissemination to underlying tissue (Trautwein-Weidner et al. 2015b; Huppler et al. 2014). Together, the tight interplay between immune cells and the epithelium guarantees for efficient fungal control in the mucosa. In addition, IL-17 may exert direct effects on *C. albicans* and thereby affect its virulence (Zelante et al. 2012).

Infections with *C. albicans* are usually not contagious. Rather, the fungus is acquired through childbirth from the mother as part of the normal microbiota. The *C. albicans* population within a given individual is largely clonal (Moorhouse et al. 2016; Bougnoux et al. 2008b; Pujol et al. 1993). The colonizing fungus

itself may turn pathogenic and cause disease when host defenses are breached. Therefore, variations in fungal isolates that exist between individuals, in addition to host factors, may contribute to disease susceptibility, although this notion is often undervalued and not well understood. *C. albicans* displays a significant intra-species diversity both at a phylogenomic and phenotypic level (Odds et al. 2007; MacCallum et al. 2009; Hirakawa et al. 2015). Phenotypic variations include differences in adherence, stress responses, gene expression, morphological plasticity and the capacity to form biofilm. The basis for this variation and its impact on the host response remain largely unclear. Strain-specific differences recently drew attention in the murine intravenous challenge model (Marakalala et al. 2013; Hirakawa et al. 2015; MacCallum et al. 2009). No study so far analyzed differences in pathogenicity of natural fungal isolates at mucosal surfaces, which is the site where *C. albicans* usually resides, and how they determine the decision between commensalism and disease.

Here, we probed the interaction between diverse and randomly selected natural *C. albicans* isolates and the host *in vivo* using the well-established model of oropharyngeal candidiasis (OPC). This allowed us to assess in a uniform and *Candida*-naïve host background the spectrum of responses that are raised through natural variations in the pathogen and how they define the outcome of the interaction with the fungus. Irrespective of their mucosal or blood origin, we observed striking differences between isolates in their ability to persist in the host, which inversely correlated with their capacity to induce a rapid inflammatory response in the host. While the accumulation of neutrophils in the infected tissue was strictly strain-dependent, the IL-17 pathway was essential across different strains to restrict fungal growth in the mucosal tissue. Strain-specific differences in the induction of an inflammatory response did not correlate with expression of known *C. albicans* virulence genes including *ECE1*, but rather associated with the capacity to cause epithelial damage and release IL-1α from keratinocytes. Together, this study emphasizes the key role of IL-17 in host defense against *C. albicans*, which is uncoupled from the neutrophil response, and it sets the stage for evaluating fungal determinants associated with commensalism versus pathogenicity of the fungus *in vivo*.

6.3 Results

6.3.1 Fungal persistence in the host varies between isolates and inversely correlates with transient weight loss of infected animals at the onset of infection.

The acute host response to OPC infection with *C. albicans* SC5314 in mice is characterized by an acute inflammatory response that comprises neutrophil infiltration and induction of IL-17 and antimicrobial effector molecules (Trautwein-Weidner et al. 2015b; Conti et al. 2009). The infection is accompanied with a transient loss in body weight. Weight recovery within 4 to 7 days generally correlates with fungal clearance in wildtype (WT) mice. In contrast, immunodeficient animals lacking these protective immune mechanisms are unable to control the fungus and suffer from prolonged cachexia (Trautwein-Weidner et al. 2015b; Conti et al. 2009).

To assess whether this response was general or affected by variation in the fungal strain, we selected a small number of *C. albicans* isolates from different origins that were genetically unrelated (**Fig S1AB)** and showed comparable growth in rich and minimal media *in vitro* (**Fig S2A**). The cell wall composition and degree of β-glucan exposure of these isolates was also comparable (**Fig S2CD**). We performed OPC infection of WT mice with four isolates, namely SC5314, Cag, 529L and 101. They were all equally capable of infecting the oral mucosa as evaluated by quantification of the fungal load in the tongue on day 1 post-infection (**Figure 7A**). Histology analysis revealed that all isolates formed hyphae in the tissue and invaded the oral keratinized epithelium (**Figure 7BC**). Transmission electron microscopy showed that they penetrated keratinocyte cell membranes in the stratum corneum comparably (**Figure 7C**). However, striking differences between the isolates were observed when analyzing the course of infection over time. The degree of weight loss induced in the mice by the different isolates varied greatly on day 1 to day 3 p.i. (**Figure 7D**). To determine how these differences were associated with fungal control in the host, we assessed the fungal burden in the tongue of infected mice on day 3 and day 7 p.i.. In contrast to strain SC5314, which was rapidly cleared to undetectable levels, all other isolates persisted in the host and high fungal counts could still be recovered one week p.i. (**Figure 7EF**).

The high fungal load remained detectable for up to 4 weeks p.i., at least in case of strain 101 (**Fig S3)** These data indicated that transient weight loss during the first days of OPC was not a consequence of high fungal burden in the mice, but rather that strong changes in weight during the initial phase of infection were associated with enhanced fungal control. Importantly, for the persistent fungal isolates no tissue pathology was observed despite the prolonged presence of high fungal loads (**Figure 7G)**.

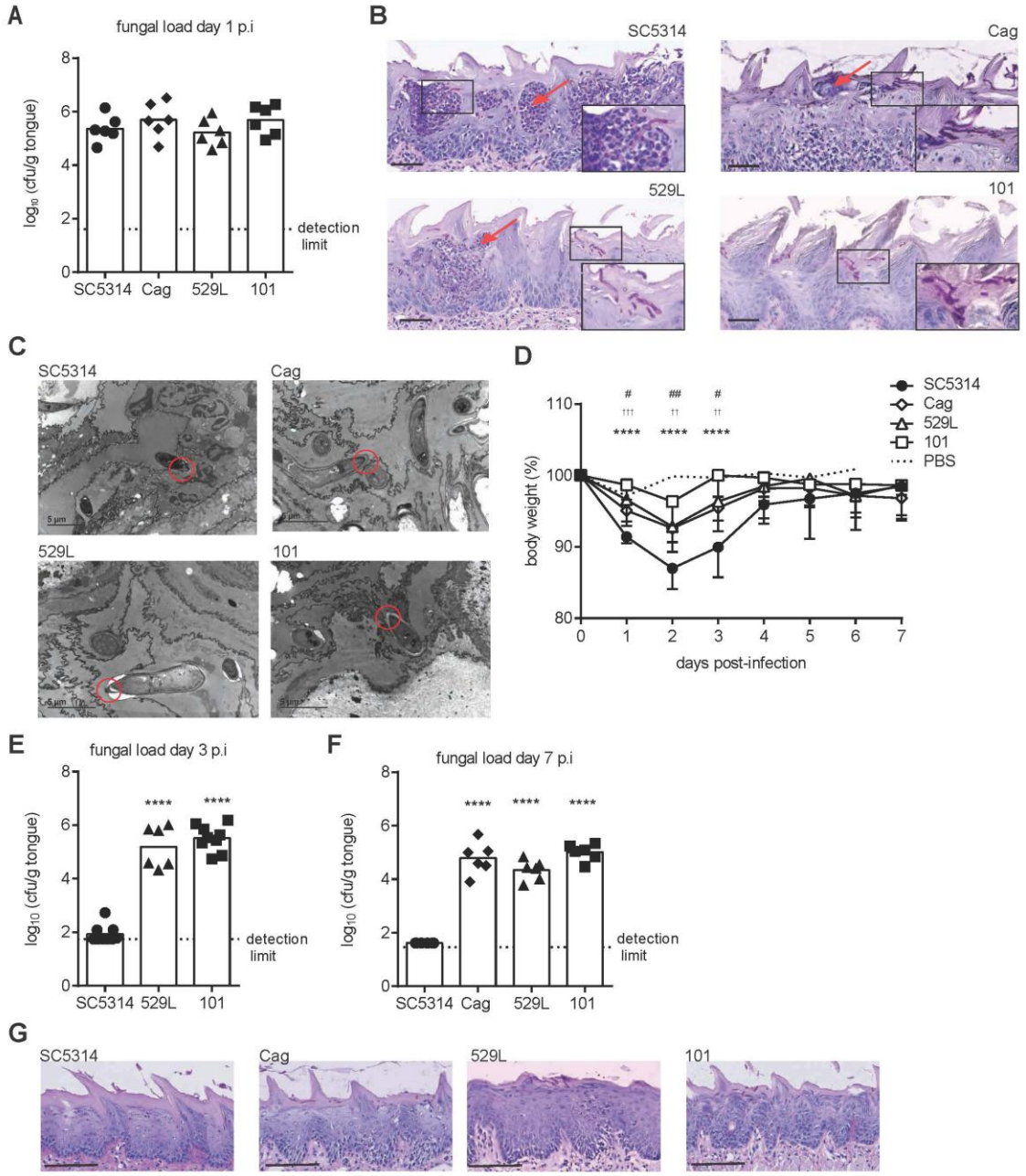

Figure 7. Fungal persistence in the host inversely correlates with transient weight loss of infected animals at the onset of infection. WT mice were infected with *C. albicans* SC5314, Cag, 529L or 101. (A) Tongue fungal burden on day 1. (B) Representative images of sagittal tongue sections (day 1) stained with PAS. Arrows: inflammatory infiltrates. Scale bars: 50 µm. Inset: 2-fold magnification. (C)

Representative transmission electron microscopy images of infected tongues (day 1). Red circles: fungal penetration of cell membranes in the stratum corneum. Scale bars: 5 µm. (D) Body weight of infected mice. Data are the mean +- SD (n = 6, pooled from two independent experiments). (E-F) Tongue fungal burden on day 3 and day 7. (G) Representative images of sagittal tongue sections (day 7) stained with PAS. Scale bars: 100 µm. In A, E, F, each symbol represents one mouse. Data are the pool of two independent experiments. The geometric mean and significant differences between each group and the SC5314-infected group are indicated. In A, E, F, each symbol represents one mouse. Data are the pool of two independent experiments. The geometric mean and significant differences between each group and the SC5314-infected group are indicated

6.3.2 Persistent fungal isolates fail to induce an acute inflammatory response in the host.

To investigate the cause of these differences in colonization versus clearance with different *C. albicans* isolates we assessed the response of the host in the four infected groups. Histology of the infected tissue revealed striking differences in the degree of inflammation induced on day 1 p.i. (**Figure 7B**). While large infiltrates of inflammatory cells, which consist mainly of neutrophils (**Fig S4A**) (Trautwein-Weidner et al. 2015b) were observed in the tongue of strain SC5314-infected mice, barely any cellular infiltrates were observed in strain 101-infected animals and only few and small infiltrates were found in the other groups on day 1 p.i.. Quantification of neutrophils by flow cytometry of single cell suspensions prepared from the infected tongues confirmed these results (**Figure 8A, Fig S4A**). Importantly, the neutrophil response to strain SC5314 was strongest, even if the infection dose was lowered 25-fold (**Fig S4B**). The blunted neutrophil response to infection with the persistent *C. albicans* isolates correlated with their limited capacity to induce neutrophil-recruiting chemokines such as CXCL2 and the granulopoietic factor G-CSF (**Figure 8BC**). G-CSF was measured in the serum accounting for the endothelial source of this factor (Altmeier et al. 2016b). Of note, we did not observe dissemination of the persistent isolates away from the mucosal epithelium despite the fact that neutrophils are critical for preventing fungal dissemination in case of strain SC5314 (Trautwein-Weidner et al. 2015b). These results demonstrate that the role of neutrophils during OPC is strain specific. Induction of IL-6 and TNF was also differentially induced in response to the different isolates, albeit difference for TNF did not reach statistical significance (**Figure 8DE**). Next, we assessed the activation of the IL-17 pathway and downstream targets on day 1 p.i.. IL-17 expression was strongly

reduced in the tongue of mice infected with the persistent *C. albicans* isolates compared to strain SC5314 (**Figure 8FG**). Similarly, *S100a9* transcripts were very weakly if at all induced (**Figure 8H**). Together, this indicated that natural *C. albicans* isolates stimulated a highly variable response in the host, whereby the extent of inflammation induction correlated with fungal clearance (see below).

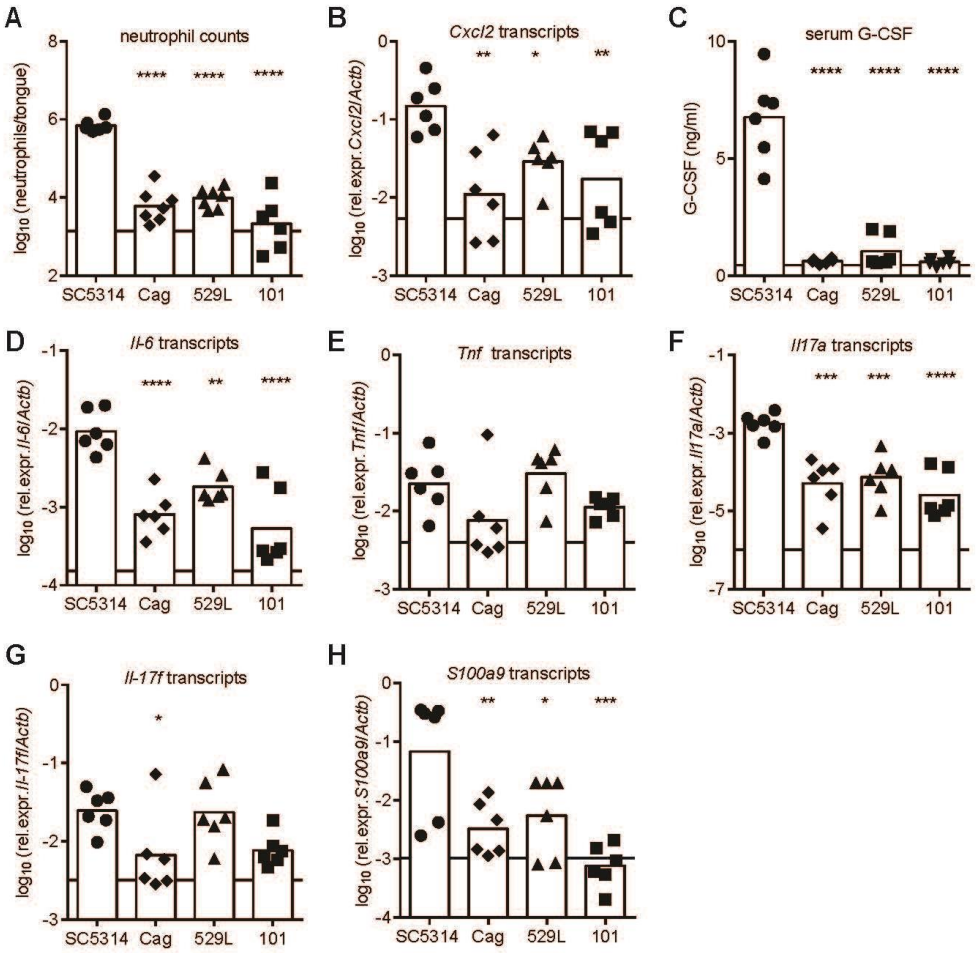

Figure 8 Persistent *C. albicans* isolates induce a limited inflammatory response in the host.
WT mice were infected with *C. albicans* SC5314, Cag, 529L or 101 and analyzed after 1 day. (A) Quantification of tongue $CD11b^+$ $Ly6C^+$ $Ly6G^+$ neutrophils by flow cytometry. (B) *Cxcl2* expression in tongue was assessed by qRT-PCR. (C) Serum G-CSF was quantified by ELISA. (D – H) *Il6*, *Tnf*, *Il17a*, *Il17f* and *S100a9* expression in the tongue was assessed by qRT-PCR. Each symbol represents one mouse. Data are the pool of two independent experiments. The mean (C) or the geometric mean (A, B, D - H) of each group and significant differences between each group and the SC5314-infected group are indicated. Solid line: (geometric) mean of a naïve control group.

6.3.3 The variation in the pathogenicity of *C. albicans* isolates does not correlate with their clinical origin

All isolates tested so far, which displayed low-pathogenicity and thus persisted in the host, were collected from the oral mucosa of patients and healthy individuals, while SC5314 was originally obtained from a blood culture. To test whether the origin of the isolates correlated with their pathogenicity, we examined the behavior of two additional independent blood isolates (UC820 and ATCC14053) in the OPC mouse model. These isolates displayed similar growth kinetics, cell wall composition and β-glucan exposure *in vitro* as strain SC5314 (**Fig S2**). Irrespective of their origin, they readily colonized the oral mucosa and persisted for over a week without inducing significant weight loss in the host (**Figure 9A-C**). Similar to the oral isolates analyzed before (**Figure 7,Figure 8**), they induced an attenuated inflammatory response (**Figure 9D-F**).

Within a larger collection of natural isolates (see below), we identified additional strains of high-pathogenicity similar to strain SC5314. One of them, strain CEC3605, which was collected from the vaginal mucosa and displayed a normal phenotype *in vitro* (**Fig S2**), was then tested for its behavior *in vivo*. Similar to SC5314, mice infected with CEC3605 displayed a strong transient weight loss (**Figure 9A**) and rapid fungal control (**Figure 9BC**). As expected from the similar behavior of CEC3605 to SC5314, CEC3605 triggered a very strong and rapid inflammatory response in the oral mucosa on day 1 p.i. with large neutrophilic infiltrates (**Figure 9DG**) and high levels of neutrophil-recruiting chemokines, *Il6* and *Tnf* transcripts in the epithelial tissue (**Figure 9H-J**) as well as high G-CSF levels in the serum (**Figure 9K**). Moreover, *Il17a, Il17f* and IL-17 target genes were strongly induced by strain CEC3605 on day 1 p.i. (**Figure 9EF, L**).

Together, these results suggested that the host compartment, from which *C. albicans* isolates were collected, was not decisive for their behavior in the host. Rather the intrinsic capacity of individual isolates to promote a response in a previously *C. albicans*-naïve host determined whether they persisted in the host or were rapidly cleared. Moreover, the degree of early inflammation induced by a strain seemed to determine the outcome of infection, with an inverse correlation between the

inflammatory response triggered in the host and fungal persistence (**Figure 9M**). This is consistent with what was observed before in different experimental systems (Rahman et al. 2007; Misme-Aucouturier et al. 2016).

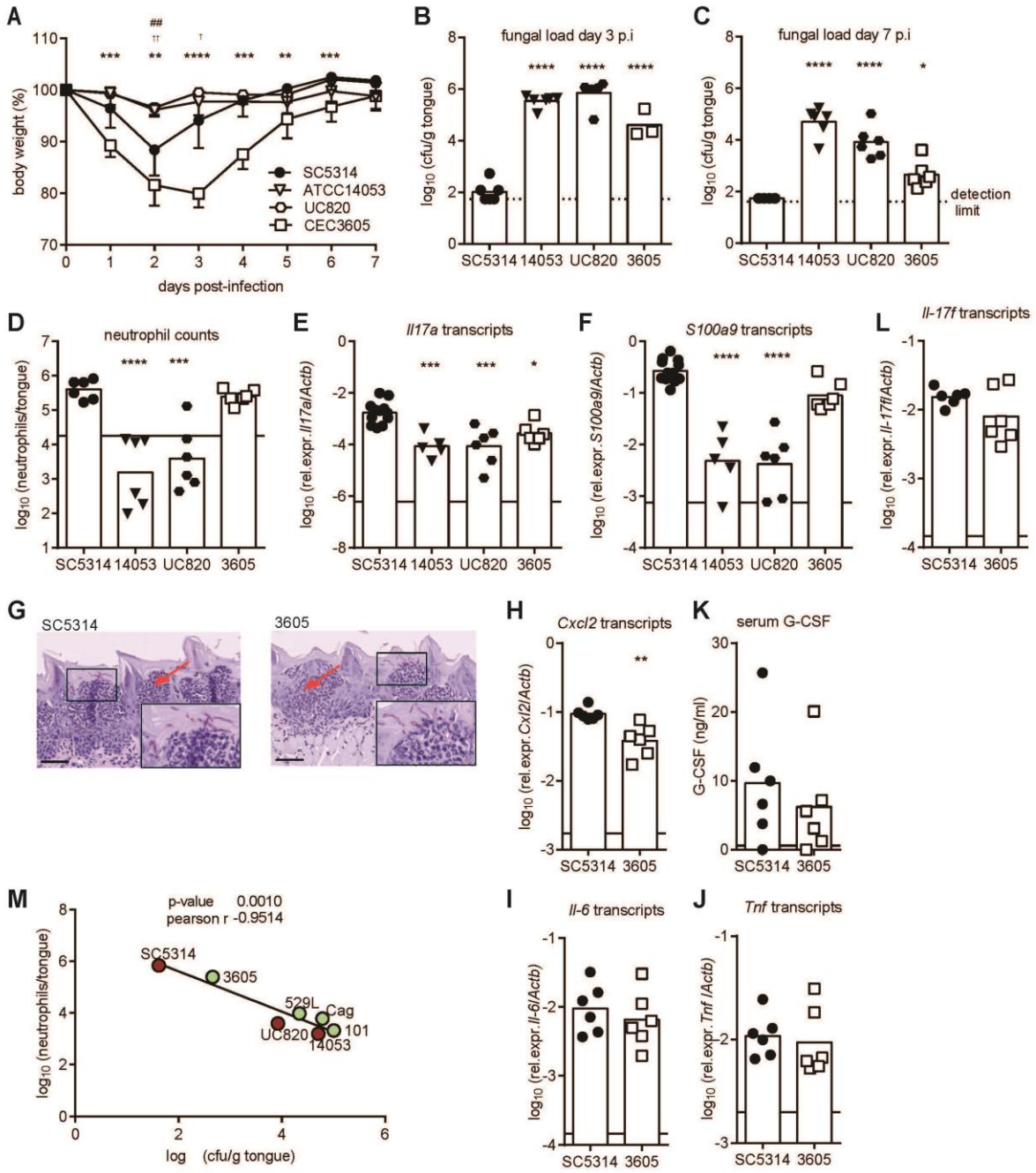

Figure 9 The variation in the pathogenicity of *C. albicans* isolates did not correlate with their origin. WT mice were infected with *C. albicans* SC5314, ATCC14053, UC820 or CEC3605. (A) Body weight of infected mice. Data are the mean + SD (n = 6, pooled from two independent experiments). (B – C) Tongue fungal burden on day 3 and day 7. (D) Quantification of tongue $CD11b^+$ $Ly6C^+$ $Ly6G^+$ neutrophils (day 1) by flow cytometry. (E – F) *Il17a* and *S100a9* expression in the tongue was assessed (day 1) by qRT-PCR. (G) Representative images of sagittal tongue sections (day 1) stained with PAS. Arrows: inflammatory infiltrates. Scale bars: 50 µm. Inset: 2-fold magnification. (H – J)

Cxcl2, *Il6* and *Tnf* expression in the tongue on day 1 was assessed by qRT-PCR. (K) Serum G-CSF on day was quantified by ELISA. (L) *Il17f* expression in the tongue was assessed (day 1) by qRT-PCR. (M) Scatter plot with data from figures 1F, 2A and 3D. Color code: mucosal isolates (green), blood isolates (red). In B – F and H – L, each symbol represents one mouse. Data are the pool of two independent experiments (except for the CEC3605 group in (B), which was from one experiment). The mean (A, K) or geometric mean (B – F, H – J, L) of each group and significant differences between each group and the SC5314-infected group are indicated. Solid line in D – F, H – L: (geo)mean of a naïve control group.

6.3.4 Filamentation *in vitro* is not a good predictor of the pathogenicity of *C. albicans* isolates in the oral mucosa *in vivo*.

To explore the underlying cause of the variability in the outcome of the interaction of the *C. albicans* isolates with the host, we compared the fungal morphology under different conditions. *C. albicans* is able to undergo morphological switching between budding yeast, pseudohyphal and hyphal forms. The ability to filament is generally thought to be a critical feature of pathogenicity (Hebecker et al. 2014). All strains formed hyphae in FCS-containing liquid medium in a comparable manner with small differences in hyphal length after 3 hours (**Fig S5A**). Differences in filamentation between the *C. albicans* isolates were much more pronounced when grown on Spider medium for 10 days. While filamentation was observed reproducibly with strains SC5314, CEC3605 and UC820, while strains Cag, 529L and ATCC14053 had a more variable phenotype and no filamentation was observed for strain 101 in this assay (**Fig S5BC**). When growing the fungal strains on TR146 keratinocytes, all isolates were found to filament, albeit the proportion of cells forming pseudohyphae or hyphae, the hyphal length and the degree of branching varied (**Fig S5D**). Importantly, no correlation was found between hyphal growth on TR146 and the behavior of the fungal isolates *in vivo*: ATCC14053, which induced a similar degree of inflammation as Cag, formed mostly short pseudohyphae, while long hyphae were observed in case of Cag (**Fig S5D**). Likewise, 101, but not UC820 formed typical hyphal structures on TR146 cells, although none of the two induced significant inflammation *in vivo* (**Fig S5D**).

Finally, we assessed fungal morphology *in vivo* using two pathogenic and two persistent isolates as examples. They all formed hyphal filaments in the infected tongue, which were hardly distinguishable in shape and length (**Figure 7B, Figure 9G**). This observation was confirmed when analyzing histology in top view of horizontally-cut tongues at 24 hours p.i. (Error! Reference source not found.**A**).

etermining the localization of *C. albicans* in the third dimension of the oral epithelium was not possible 1 day p.i. when large neutrophil infiltrates were present in the mucosa of mice infected with SC5314 and CEC3605 (**Figure 7B,Figure 9G**). We therefore shifted our analysis to an earlier time point during infection. This revealed important differences in the invasion depth of *C. albicans* isolates in the oral epithelium, which reflected the differential inflammatory response that they elicited (Error! Reference source not found.**B**). While the pathogenic isolates SC5314 and CEC3605 penetrated into the stratum spinosum and granulosum, the persistent isolates 529L and 101 were found exclusively in the amorphous stratum corneum at this early (Error! Reference source not found.**B**) as well as at later time points (**Figure 7G**). Together, these results indicated that the variation in pathogenicity between *C. albicans* isolates were not easily explained by differences in fungal morphology, but rather by their differential localization within the squamous mucosal epithelium.

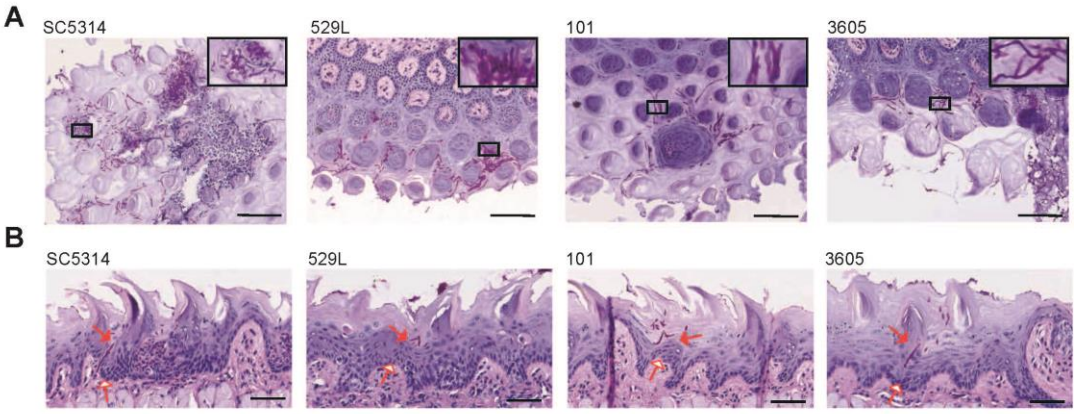

Figure 10 Filamentation *in vitro* is not a good predictor of the pathogenicity of *C. albicans* isolates in the oral mucosa *in vivo*. Representative images of tongue sections from WT mice infected with SC5314, 529L, 101 or CEC3605 stained with PAS. Top view of dorso-ventral sections on day 1 (A) and sagittal sections after 12 hours (B). Inset in A: 4-fold magnification. Filled arrows: boundary between stratum corneum and stratum granulosum; open arrows: stratum basale.

6.3.5 Induction of damage and IL-1α in keratinocytes reflects the pathogenicity of *C. albicans in vivo*.

As a first contact point, epithelial cells play a key role in the initial interaction between *C. albicans* and the host during superficial infections. *C. albicans* can adhere, invade and damage the epithelium, all parameters associated with enhanced virulence (Hebecker et al. 2014). All isolates adhered to the

keratinocytes cell line TR146, albeit to variable degrees (**Fig S6A**). Likewise, the capacity of the isolates to form biofilm varied and did not show a pattern that could be associated with the observed phenotypes *in vivo* (**Fig S6B**). In contrast, the extent to which the different isolates caused epithelial cell damage, as assessed by LDH release, reflected their differential induction of inflammation *in vivo* (**Figure 11AB**). Importantly, the differences in damage induced by the isolates were conserved at different infection doses (**Fig S6C**). The release of LDH by cultured keratinocytes in response to *C. albicans in vitro* was thus a good correlate for fungal pathogenicity *in vivo* (**Figure 11CD**).

In the following, we used the LDH release assay as a surrogate to examine the variation in pathogenicity of *C. albicans* within a larger collection of 74 natural isolates. Although largely diverse, they all ranked between the two extremes identified as high damage inducers (>10% LDH release after 8 hours of stimulation, exemplified by SC5314 and CEC3605) and low damage inducers (<10% LDH release after 24 hours of stimulation, exemplified by 101, 529L and UC820). A small proportion of isolates fell into the group of intermediate damage inducers (<10% LDH release after 8 hours, >10% after 24 hours of stimulation) (**Figure 11E**). Confirming our previous results **(Figure 9M**), the degree of damage induced by different isolates did not correlate with their source, namely mucosal tissues or blood cultures (**Figure 11F**).

The induction of cellular damage by *C. albicans* was recently associated with the virulence factor *ECE1*, which gives rise to a pore forming toxin Candidalysin (Moyes et al. 2016). We therefore assessed *ECE1* expression by *C. albicans* isolates in contact with TR146 cells. Although we detected significant differences in *ECE1* expression between the different isolates (**Figure 11G**), they did not correlate with the degree of LDH release (**Figure 11H**) or the *in vivo* pathogenicity induced. Moreover, we did not detect significant differences in expression of *KEX1* and *KEX2*, two proteinases involved in the processing of *ECE1* (Moyes et al. 2016) between the isolates tested (**Fig S6D**). Therefore, mechanisms beyond those involving *ECE1* seem to determine the capacity of *C. albicans* to cause host damage when moving away from strain SC5314. Expression of other well-known virulence factors, such as *ALS3,*

SAP4, SAP5, SAP6, SAP9 and *HWP1* and the virulence-associated transcriptional regulators *EFG1* and *CPH1* were also not significantly altered between the isolates (**Fig S6EF**).

Induction of cellular damage is linked to the release of cytoplasmic molecules, some of which can act as alarmins and promote inflammation if freed into the extracellular space where they do not usually reside. Indeed, IL-1α release by keratinocytes in response to *C. albicans* isolates mirrored the levels of LDH release (**Figure 11IJ**). In contrast, keratinocyte secretion of other cytokines such as IL-6 and IL-8 did not follow the same pattern (**Fig S6G**). The differential IL-1α response by pathogenic and non-pathogenic isolates was also observed in the infected tongue, as shown for a prototypic isolate of each type, SC5314 and 101 (**Figure 11K**).

Together, these data indicate that the induction of damage and release of danger-associated molecules from cultured keratinocytes *in vitro* is a good predictor of *C. albicans* pathogenicity *in vivo*. Moreover, they suggest that the epithelium translates differences in the fungus into differential host responses.

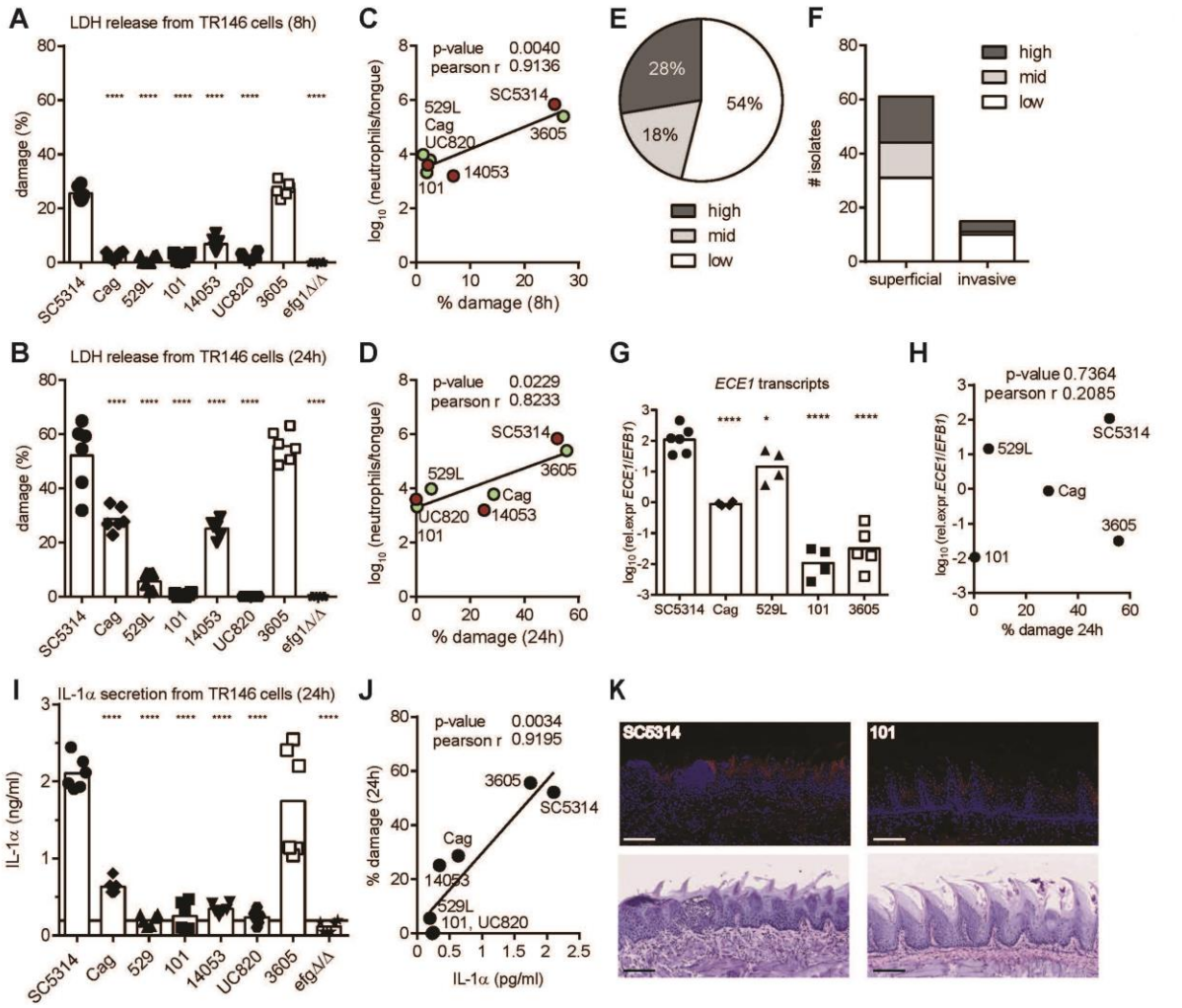

Figure 11 Induction of damage and IL-1α in keratinocytes reflects the pathogenicity of *C. albicans in vivo*. (A – B) LDH release from TR146 cells stimulated with *C. albicans* SC5314, Cag, 529L, 101, ATCC14053, UC820, CEC3605 or the mutant strain efg1Δ/Δ, which was included as a negative control, for 8 hours (A) or 24 hours (B). (C – D) Scatter plot of data from figures 5A, 2A and 3D (C) or figures 5B, 2A and 3D (D). Color code as in figure 3M. (E – F) Damage induction by *C. albicans* 76 isolates (including the seven isolates used in this study) classified as low (<10% LDH release at 24 hours), mid (<10% at 8 hours / >10% after 24 hours) and high (>10% after 8 hours). In F, the number of isolates in each category is shown separately for isolates from mucosal surfaces (superficial) and blood cultures (invasive). (G) *ECE1* mRNA expression by *C. albicans* isolates upon contact with TR146 keratinocytes for 24 hours. (H) Scatter plot of data from figure 5B and G. (I) IL-1α levels in the samples from (B). Solid line: basal IL-1α levels in the supernatant of unstimulated TR146 cells. (J) Scatter plot of data from figure 5B and I. (K) Representative images of sagittal tongues sections from WT mice infected with SC5314 or 101 for 1 day and stained with anti-IL1-α (red) and DAPI (blue) (top) or PAS (bottom). Scale bars: 100 µm. In A, B and I, each symbol represents a separate well, the mean of each group is indicated. Data are a pool of two independent experiments. In G, data are the pool of 3 independent experiments, the geomean of each group is indicated.

6.3.6 Persistent *C. albicans* isolates induce a delayed and qualitatively altered response in the oral mucosa

Our data suggested that non-pathogenic *C. albicans* isolates persisting in the stratum corneum of the oral mucosa induce a very limited host response. However, the observation that they did not overgrow the host or disseminate, indicated that they were not completely uncontrolled by the host. We therefore examined the antifungal response to the persistent strains 101 and 529L at later time points. In contrast to the strong and transient neutrophil response induced by pathogenic isolates (**Figure 12A**), 529L and 101 induced small and isolated neutrophil infiltrates in the infected tongue on day 3 and 5 p.i. (**Figure 12A**). This attenuated response was paralleled by a strongly impaired induction of G-CSF and *Cxcl2* expression (**Figure 12BC**).

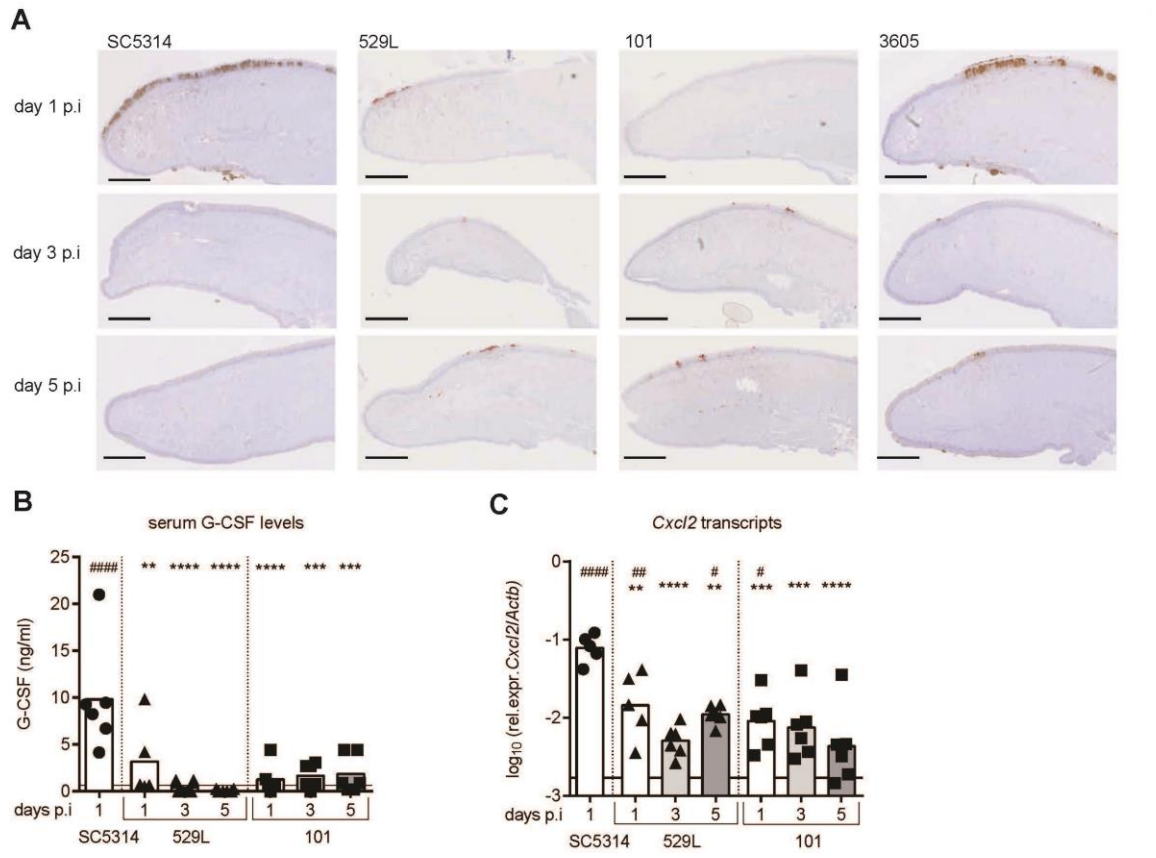

Figure 12 The neutrophil response to persistent isolates is strongly attenuated. (A) Representative images of sagittal tongue sections from WT mice infected with SC5314, 529L, 101 or CEC3605 for 1, 3 or 5 days and stained with anti-Ly6G. Scale bars: 1 mm. (B – C) Serum G-CSF (B) and tongue *Cxcl2* mRNA (C) of WT mice infected with SC5314, 529L or 101 for 1, 3 or 5 days. Each symbol represents one mouse. The mean (B) or geometric mean (C) of each group is indicated. Data in B and C are pooled from two independent experiments. Solid line: (geo) mean of a naïve control group.

Significant differences between each group and the SC5314-infected group (day 1) or between naïve and infected groups for each strain are indicated by asterisks or by hashes, respectively.

In contrast, expression of IL-17 and IL-17 target genes was strongly induced in the tongue of 529L- and 101-infected mice on day 3 and 5 that reached comparable levels to those found in SC5314-infected tongues on day 1 p.i. (**Figure 13A-C**). These data were further confirmed by measuring IL-17A and IL-17F protein induction in response to persistent isolates at later time points (**Figure 13DE)**. Likewise, *C. albicans*-specific Th17 cells were induced comparably by all isolates tested in the draining lymph nodes on day 7 p.i. (**Fig S7**).

Together, these results indicated that *C. albicans* isolates, which initially induced a restrained response, did not remain undetected by the immune system and an IL-17 signature response was readily triggered after a few days of fungal presence in the host. In contrast, the recruitment of neutrophils remained very limited, even at later time points when the fungal load was still high.

6.3.7 IL-17 signaling is essential for preventing fungal overgrowth irrespective of the degree of fungal pathogenicity

To evaluate whether the IL-17 response to OPC infection with persistent strains was relevant for host defense and fungal control in the oral mucosa despite the delayed induction, we infected mice with a genetic defect in IL-17 signaling. Both IL-17RA- and IL-17RC-deficient mice showed increased fungal loads after infection with strains 101 and 529L if compared to WT control hosts (**Figure 13FG**). Groups of mice infected with strain SC5314 were also included in this experiment for comparison (**Figure 13FG**). The relevance of the IL-17 pathway for limiting the growth of all fungal isolates was also obvious when analyzing infected tongues by histology (**Figure 13H**). Infection of IL-17 receptor deficient mice with strains 101 or 529L did not results in major changes in body weight (**Fig S8**), re-emphasizing that the weight loss during the early phase of OPC, as seen with pathogenic strains of *C. albicans*, was not directly associated with the fungal burden.

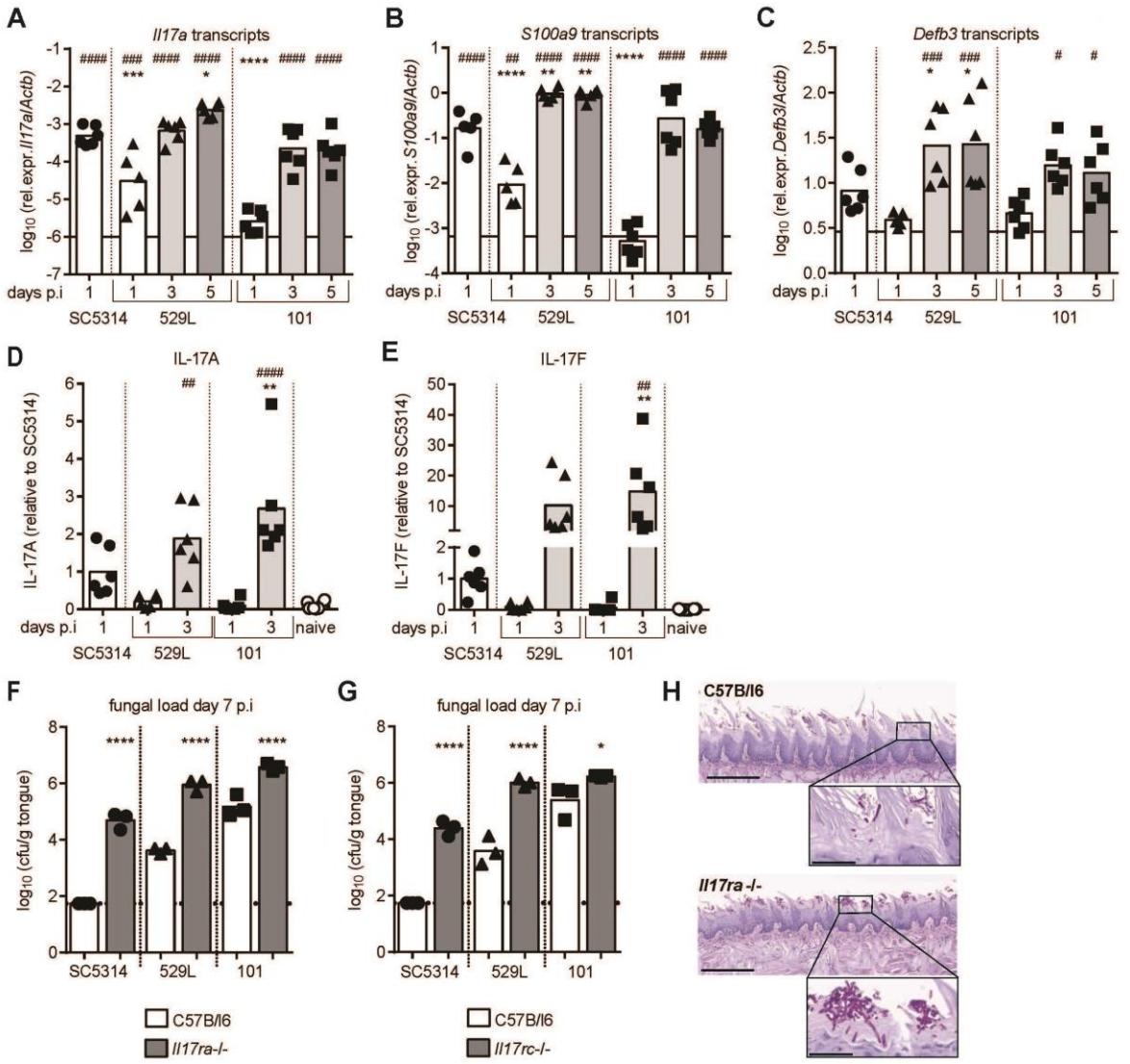

Figure 13 IL-17 signaling is essential for preventing fungal overgrowth irrespective of the degree of fungal pathogenicity (A – C) *Il17a*, *S100a9*, *Defb3* expression in the tongue of WT mice infected with SC5314, 529L or 101 for 1, 3 or 5 days. Each symbol represents one mouse. The geometric mean of each group is indicated. Solid line: geometric mean of a naïve control group. Data are pooled from two independent experiments. (D – E) IL-17A and IL-17F protein levels in the tongue of WT mice infected with 529L or 101 for 1 or 3 days compared to the respective levels induced by SC5314 on day 1 post-infection. In A-E, significant differences between each group and the SC5314-infected group (day 1) or between naïve and infected groups for each strain are indicated by asterisks or by hashes, respectively. (F – G) Tongue fungal burden on day 7 in WT and *Il17ra*-/- (F) or *Il17rc*-/- mice (G) infected with SC5314, 529L or 101. Each symbol represents one mouse. The geometric mean of each group is indicated. Significant differences of the knockout to the corresponding WT control group for each fungal strain are indicated. (H) Representative images of sagittal tongue sections of 101-infected WT and *Il17ra*-/- mice stained with PAS. Scale bars: 250 µm. Inset: 4 fold magnification, scale bars: 50 µm.

In conclusion, these results showed that IL-17 signaling plays a non-redundant role for mucosal immunity against *C. albicans*, irrespective of the nature of the infecting strain, whereas epithelial cell

damage, mucosal inflammation and neutrophil infiltration display a strain-specific dichotomy and are associated with fungal pathogenicity.

6.4 Discussion

C. albicans is a common pathobiont and one of the most important disease-causing fungi in humans. Disease symptoms occur if host defenses are breached. Specific immune mechanisms that protect from different forms of diseases have been characterized. However, little attention has been paid so far to the contribution of the natural diversity of *C. albicans* to the decision between health and disease. In this study, we took advantage of a mouse model of OPC to probe the differential interaction of the host with diverse fungal isolates in a complex but uniform and *C. albicans*-naïve environment *in vivo*. We identified major qualitative and temporal differences in immune activation by distinct fungal isolates. The capacity of *C. albicans* to trigger a response by the epithelium such as the release of alarmins/IL-1α correlates with the induction of an inflammatory response and the rapid control of the fungus by the host. Thereby, the functional variability of *C. albicans* natural isolates determines a fine balance between commensalism and pathogenicity and thus the long-term outcome of the interaction with the host. In contrast, the requirement of the IL-17 pathway for preventing fungal outgrowth was conserved among all isolates tested, highlighting the key role of this cytokine in host protection from *C. albicans*, irrespective of the fungal pathogenicity.

Significant diversity exists between natural isolates of *C. albicans*, both from a phylogenomic as well as a phenotypic perspective. Population studies of *C. albicans* support a predominantly clonal mode of reproduction of this diploid and largely heterozygous organism (Pujol et al., 1993; Bougnoux et al., 2008b; Moorhouse et al., 2016). Examples of phenotypic variation across *C. albicans* isolates have been studied most extensively in the context of antifungal drug resistance (Cowen et al., 2015; Loll et al., 2015). Differences between natural *C. albicans* isolates that affect their interaction with the host have also been reported (MacCallum et al., 2009). A striking example of phenotypic intraspecies variation is provided by the differential behavior of two unrelated natural isolates in mice lacking the β-1,3-glucan

receptor Dectin-1, which was based on differences in the composition and nature of their cell walls and recognition by innate immune cells *in vivo* (Marakalala et al., 2013). Variation may also exist in the extent to which TLR4 recognizes different *C. albicans* isolates (Netea et al., 2010). In all these examples, the genetic basis for the phenotypic differences remains unexplored. This also applies to the isolates analyzed in this report. Future research will be needed to decipher the genetics underlying natural differences in the virulence traits of *C. albicans*, although the high degree of sequence variation between individual isolates makes this endeavor a challenge (Ford et al., 2015; Hirakawa et al., 2015). Existing intraspecies variation however is likely contributing to the probability of disease development and/or the severity of symptoms when comparing individuals with a comparable immune status. *C. albicans* infections are generally not transmissible, but rather caused by the fungal strain that was already present as a component of the microbial flora prior to the outbreak of symptoms. This is consistent with the observation that the origin of the isolates from mucosal surfaces or blood cultures did not segregate by differences in pathogenicity.

In the current study, we focused on how the fungal diversity affects the host response *in vivo*. Infection of mice via the sublingual route revealed a spectrum of temporally and qualitatively different immune responses to different isolates with the two extremes exemplified by strains SC5314 and 101, and various intermediate cases. While some isolates triggered a massive infiltration of neutrophils and a strong expression of IL-17 in the infected tissue within one day p.i., as it was observed previously for strain SC5314 (Gladiator et al., 2013; Trautwein-Weidner et al., 2015b), the response to other isolates was delayed with induction of IL-17 from day 3 p.i., but only very limited neutrophil recruitment. The former scenario was associated with rapid fungal elimination from the host, whereas the latter allowed persistent colonization of the host mucosal epithelium by the isolate. Rapid induction of an inflammatory response appeared thus to be critical for the decision between fungal colonization versus elimination and the contribution of infiltrating neutrophils to the response is strain-dependent. The segregated host response was paralleled by very distinct patterns of weight loss and recovery during infection with different isolates. Our data suggest that not the fungal load, as previously thought

(Conti et al., 2009; Trautwein-Weidner et al., 2015b), but rather the rapid inflammatory response, and possibly the associated damage caused to the host, elicits the drop in weight on day 1 – 2 p.i.. It remains unclear whether and which specific host factor(s) account for it, and if so, which one. IL-1 or TNF, which can cause cachexia in other contexts, do not seem to be involved in the experimental setting used here. According to our preliminary data, IL-1 receptor deficiency did not relieve the drop in weight, and no significant increase in systemic TNF levels were measured upon infection with strain SC5314.

The qualitative differences in the host response between different *C. albicans* isolates, and in particular the observation that the delayed induction of IL-17 by persistent isolates was not accompanied by significant neutrophil infiltration, underlines the notion that the two pathways are uncoupled during OPC (Trautwein-Weidner et al., 2015b). Although genes associated with neutrophil trafficking and function can be affected by IL-17 in some cases (Schwarzenberger et al., 1998; Laan et al., 1999; Miyamoto et al., 2003), defective IL-17 signaling does not impair neutrophil recruitment to the site of infection during OPC (Trautwein-Weidner et al., 2015b), and the induction of IL-17 does not entail an enhanced neutrophil response as shown here.

The IL-17 pathway has emerged as a key player in protection from superficial candidiasis, as evidenced by the strong association of CMC with defects in genes linked to IL-17 production or signaling (Puel et al., 2012). Likewise, the critical role of the IL-17 pathway in preventing uncontrolled fungal overgrowth in the epithelium was conserved in mice irrespective of the pathogenicity of the infecting strain, albeit the delayed induction of IL-17 and IL-17 target genes appeared insufficient from eliminating colonizing fungus from the murine host.

C. albicans-specific Th17 cells can be found in the circulation of most individuals irrespective of the fungal diversity within the human population (Sparber and LeibundGut-Landmann, 2015). *C. albicans*-specific Th17 cells are also induced in mouse models of superficial candidiasis (Bär et al., 2012; Hernandez-Santos et al., 2012; Trautwein-Weidner et al., 2015a). In this study we compared the Th17 response to different *C. albicans* isolates and found that it was induced to comparable degrees in all cases. Thus, the drastic differences in inflammation during the initial acute host response do not

translate in differences of adaptive immunity. This suggests that the initial inflammatory response to some isolates is redundant for the initiation of adaptive antifungal immunity. Alternatively, the increased antigen load in case of infection with persistent isolates may also compensate for the initially limited induction of T cell activating/polarizing cytokines.

During OPC infection with highly virulent *C. albicans* strains, such as SC5314, IL-17 is induced rapidly and prior to the initiation of cytokine production by conventional Th17 cells (Gladiator et al., 2013; Conti et al., 2014; Kashem et al., 2015). Lymphocytes of the innate immune system including innate TCR$\alpha\beta^+$ T cells, TCR$\gamma\delta^+$ T cells and innate lymphoid cells (ILCs) have been identified as the source of the immediate production of IL-17 in the skin and oral mucosa within one day p.i. (Gladiator et al., 2013; Conti et al., 2014; Kashem et al., 2015). The cell type(s) responsible for tongue IL-17 production 3 – 5 days after infection of mice with colonizing isolates of *C. albicans* and the signals responsible for the delayed IL-17 production in this situation remain to be identified.

Our data demonstrated that the differential induction of an inflammatory response by diverse *C. albicans* isolates in the oral mucosa correlated closely with the degree of damage that these isolates caused in keratinocyte cultures. Consistent with previous reports (Dongari-bagtzoglou et al., 2004; Moyes et al., 2016), LDH release as a readout for damage induction also correlated closely with the release IL-1α by keratinocytes. We show that this applies also *in vivo*, where induction of IL-1α in the oral epithelium was greatly increased in response to strain SC5314 compared to strain 101. IL-1 signaling is linked to the regulation of neutrophil recruitment in response to *C. albicans* (Tomalka et al., 2015; Altmeier et al., 2016). Therefore, the epithelium is capable of sensing differences between fungal isolates that it translates into an inflammatory response, and thereby determines the overall degree of the (innate) host response. The measure of LDH and IL-1α release by keratinocytes in response to *C. albicans* isolates thus serves as a surrogate for determining the degree of inflammation induced in the host tissue *in vivo* and the efficiency of fungal control, which in turn are linked to differences between commensalism and pathogenicity. The keratinocyte assay may thus be used to predict the outcome of the interaction between *C. albicans* isolates and the host epithelial/superficial

tissues *in vivo*. It may serve for screening collections of fungal isolates from cohorts of patients with specific immune defects to assess a possible correlation between the pathogenicity of the isolates and the severity of symptoms.

Epithelial damage caused by *C. albicans* appears to result from a combination of different mechanisms. Filamentation is generally thought to be a prerequisite for damage induction, and expression of many virulence genes is associated with fungal morphology (Thompson et al., 2011). More recent results however have challenged the dogma that there is a precise correlation between morphology and virulence/damage, and filamentation *per se* appears not sufficient for damage induction or virulence (Noble et al., 2010; Moyes et al., 2016). Similarly, the degree of filamentation of different *C. albicans* isolates analyzed in this study did not correlate well with the degree of damage they induced in keratinocytes. Moreover, the expression of well-known virulence genes such as *ALS3*, *SAP4, SAP5, SAP6, SAP9*, *HWP1* and *EFG1* upon fungal contact with the epithelium did also not match the functional response of the epithelium to the different isolates. Among all examined virulence genes, the largest variations in expression between the isolates were observed for *ECE1*. Proteolytic cleavage of the Ece1 protein leads to the release of a peptide toxin called Candidalysin, which was recently described to be sufficient and required for induction of epithelial cell damage by *C. albicans* strain SC5314 (Moyes et al., 2016). While we could confirm strong expression of *ECE1* by strain SC5314 in contact with keratinocytes, *ECE1* expression did not correlate with keratinocyte damage induction in all cases. A limitation of our study may be that *ECE1* expression was measured at the transcript level, and polymorphisms in the *ECE1* gene were not analyzed, which may add to the complexity in the system. The molecular and genetic basis for the diversity among *C. albicans* isolates triggering epithelial cell damage and inflammation, and thus for the variation between pathogenicity and commensalism in the host remains to be determined in the future. Importantly, of all the different parameters analyzed *in vitro* in this study, only very few could be linked closely to the behavior of *C. albicans* isolates *in vivo* in the living host organism. Results from *in vitro* studies, such as the assessment of fungal growth on Spider medium, should thus be interpreted with care and not extrapolated to fungal pathogenicity *in*

vivo. This is reminiscent of strain-specific differences in virulence that manifest in Dectin-1-deficient hosts, which could only be revealed *in vivo* (Marakalala et al., 2013).

Together, our extensive study with different unrelated *C. albicans* isolates in contact with the host offered detailed insights into the intricate interplay between the fungus and host *in vivo*, and emphasized the limitations of results obtained from studies with a single (random) strain. This study taught us how not only different immune pathways differ in their contribution to antifungal immunity, but also how variations in the fungus result in selective activation of distinct defense mechanisms and thereby influence the outcome of the microbe-host interaction. While the requirement of IL-17 for fungal control in the mucosa is highly conserved, inflammation and neutrophil infiltrates are only triggered by highly pathogenic strains, which thereby induce their rapid clearance. Accordingly, the natural diversity of *C. albicans* is an important determinant of the fine balance between commensalism and pathogenicity *in vivo*.

6.5 Material and Methods

6.5.1 Fungal strains and media

The *C. albicans* blood isolates SC5314 (Gillum et al. 1984a), UC820 (ATCC MYA-3573 (Lehrer, Cline 1969)), ATCC14053 (Skowronski, Feldman 1989), the oral isolate 529L (isolated from an oral candidiasis patient (Rahman et al. 2007), a kind gift from J. Naglik), the oral isolates 101 (isolated from a healthy volunteer during an epidemiological clinical trial (approved by the cantonal ethics committee of the Canton of Ticino, Switzerland, approval number CE 2669)), Cag (isolated from a CMC patient with unknown etiology, kindly obtained from D. Firinu) and the vaginal isolate CEC3605 ((Sdoudi et al. 2014), isolated from a healthy carrier) were included in this study. Additional 67 isolates of different origin (mainly Europe and North Africa) were from the collection of the d'Enfert laboratory (Sdoudi et al. 2014; Bougnoux et al. 2006; Odds et al. 2007; Bougnoux et al. 2008a; Shin et al. 2011; Angebault et al. 2013). *C. albicans* isolates were obtained after written informed consent by adult subjects or by their

parent/guardian in case of children and were analyzed anonymously. The isogenic mutant strain efg1Δ/Δ ((Lo et al. 1997a), a kind gift from A. Mitchell) was used as a control in some experiment.

All isolates were maintained on Sabouraud or Yeast Peptone Dextrose (YPD) agar plates for short term and in glycerol at -80°C for long term storage. For determining fungal growth *in vitro*, Yeast Nitrogen Base (YNB) with 2% Glucose or Hams`s Nutrient Mixture F12 medium (SIGMA) supplemented with L-Glutamine, Penicillin, Streptomycin and 1% FCS was used. For other *in vitro* experiments and for *in vivo* and cell culture infection experiments, *C. albicans* was inoculated at an OD_{595} = 0.1 and grown in YPD medium at 30°C and 180 rpm for 15-18 hours. For induction of hyphae in liquid medium, Hams`s Nutrient Mixture F12 medium (SIGMA) supplemented with L-Glutamine, Penicillin, Streptomycin and 10% FCS (filamentation assay) or 1% FCS (RNA-Isolation, damage induction) was used. For determination of the fungal cell wall composition and β-glucan exposure, *C. albicans* was grown in YPD medium at 37°C overnight and subcultured into fresh YPD at OD_{600} = 2 for 3.5 hours at 37° C to obtain logarithmic-growing cells.

6.5.2 Microsatellite analysis

For microsatellite analysis, *C. albicans* was grown overnight on Sabouraud agar plates at 37°C and DNA was extracted using InstaGene matrix following the manufacturer's instructions. Primers were *Cdc3* fwd, 5'-CAGATGATTTTTTGTATGAGAAGAA-3', *Cdc3* rev 5'-CAGTCACAAGATTAAAATGTTCAAG-3', *Ef3* fwd 5'-TTTCCTCTTCCTTTCATATAGAA-3', *Ef3* rev 5'-GGATTCACTAGCAGCAGACA-3' , *His3* fwd 5'-TGGCAAAAATGATATTCCAA-3', *His3* rev 5'-TACACTATGCCCCAAACACA-3', *CaV* fwd 5'-TGCCAAATCTTGAGATACAAGTG-3', *CaV* rev 5'-CTTGCTTCTCTTGCTTTAAATTG-3', *CaVII* fwd 5'-GGGGATAGAAATGGCATCAA-3', *CaVII* rev 5'-TGTGAAACAATTCTCTCCTTGC-3' (Botterel et al. 2001; Sampaio et al. 2005). One primer of each set was 5' labelled with Hexachloro-fluorescein (for CDC3, HIS3 and CAVII) fluorescein amidite (for EF3, CAV), respectively. PCR reactions were done according to standard protocols using HotStarTaq Polymerase (Qiagen) and a 2720 Thermal Cycler (Applied Biosystems). Gene products were analyzed on a 3500 Genetic Analyzer (Applied Biosystems) using a

GeneScan 600 Liz dye size standard (Applied Biosystems) and GeneMapper v4.1 software (Applied Biosystems).

6.5.3 Multilocus sequence typing (MLST)

MLST was performed as published previously (Bougnoux et al., 2003). Allele and diploid sequence type (DST) assignments were determined using the *C. albicans* MLST database (http://pubmlst.org/calbicans). A UPGMA dendrogram was constructed using the Molecular Evolutionary Genetics Analysis (***MEGA***) v7 software.

6.5.4 *C. albicans* biofilm formation

Biofilm formation was assessed as described (Nobile et al. 2006) with the following modifications. After overnight culture in YPD, *C. albicans* yeast cells were washed twice in PBS and resuspended at 10^6 cells/ml in RPMI medium (Sigma) supplemented with 10% fetal calf serum (FCS). The cell solution was added to 1 cm^2-sized FCS-treated adhesive silicone gel sheets (Smith & Nephew) placed into a 24-well cell culture plate and incubated for 90 minutes at 37° C, 60 rpm for initial cell adhesion. Silicone gel sheets were then washed with PBS to remove non-adherent cells and moved to a new 24-well plate containing 1ml fresh medium. After 48 hours at 37° C in a humidified incubator without shaking, the squares were washed with PBS and biofilm was assessed at OD_{490} after addition of 0.5g/l filtered XTT (Sigma) in PBS.

6.5.5 *C. albicans* filamentation assays

After overnight culture in YPD, *C. albicans* yeast cells were washed twice in PBS and adjusted to 10^7 cells/ml in Hams`s Nutrient Mixture F12 medium (SIGMA) supplemented with L-Glutamine, Penicillin, Streptomycin and 10% FCS. One ml of this cell suspension was incubated for 3 hours at 37°C on a glass coverslips placed in a 24-well plate. After three time washing with PBS, cells were stained with Calcofluor white. Hyphae length was determined with an Axiolab Microscope (Carl Zeiss) and AxioVisionRel4.6 software.

For testing the colony morphology on Spider medium *C. albicans* cells were grown in YPD and washed in PBS as described above, and adjusted to 10^7 cells/ ml in PBS. 5µl of this cell suspension and of a 1:100 dilution was spotted on Spider medium plates and incubated for 10 days at 37°C. Pictures were taken with a Dinolite Digital Microscope and the Dinocaptire 2.0 software.

6.5.6 Analysis of the fungal cell wall composition

Logarithmically-growing *C. albicans* was washed three times in 50ml of 1mM cold phenylmethanesulfonyl fluoride (PMSF). 3 volumes of glass beads were then added to one volume of pellet and vortexed for 10-15 times at 1-minute intervals to break the cells. The cell lysate was again washed three times with 50 ml cold PMSF, before addition of 3 volumes of 2% SDS to the pellet and incubation for 10 min at 100° C for two rounds. SDS was removed by washing the pellet in 1mM PMSF and the pellet was dried overnight at 55°C before re-suspension in 1mM PMSF to a solution of 0.01 g dry weight / ml and storage at -20°C. Total chitin was extracted and measured as described (Kapteyn et al. 1997). Total mannan were extracted from isolated cell walls by alkali treatment and further precipitated with Fehling's solution as a copper complex as described (Chaffin et al. 1998). Total glucan was extracted as described (Dijkgraaf et al. 1996) and the extracted glucan and mannan components were then measured by the Dubois method (DuBois et al. 1956) using glucose as a standard.

6.5.7 Determination of β-glucan exposure of *C. albicans*

Quantification of β-1,3-glucan exposure on the surface of *C. albicans* was done as previously published (Wartenberg et al. 2014), with some modifications. 200 µl of logarithmically-growing *C. albicans* suspension was pelleted in a 96-well plate by centrifugation at 3500 rpm for 5 minutes. Cells were fixed with 1% formaldehyde, washed twice with PBS and blocked with 1% BSA for 1 hour at room temperature with gentle shaking, before incubation with an anti-β-1,3-glucan monoclonal antibody (mouse IgG, Biosupplies; 1:600 dilution in 1% BSA) overnight at 4° C with gentle shaking, followed by three washing steps in PBS, incubation with an Alexa Fluor 488-coupled anti-mouse IgG (Molecular Probes; 1:300 dilution) for 1 hour at room temperature with gentle shaking and three final washing

steps in PBS. Relative fluorescent intensities were then calculated by taking the ratio of the tested sample stained with/without secondary Alexa Fluor 488-coupled anti-mouse IgG antibody.

6.5.8 Cell lines

The oral keratinocyte cell line TR146 ((Rupniak et al. 1985), Sigma) was grown in Hams`s F12 nutrient mixture supplemented with L-Glutamine, Penicillin, Streptomycin and 10% FCS if not indicated otherwise. For experiments, cells were seeded at $2x10^5$ cells/well in 6-well plates or at $2x10^4$ cells/well in 96-well plates, respectively, and grown to confluent monolayers for 2-3 days prior to infection as described below for the individual assays.

6.5.9 Filamentation of *C. albicans* on TR146 cells

For assessing *C. albicans* filamentation, TR146 cells were grown to confluency on cover slips placed in a cell culture dish. *C. albicans* was grown in YPD overnight, washed twice in PBS and added onto the keratinocytes in cell culture medium at 10^3 cells/ml. After 24 hours, the medium was removed and the cells fixed with acetone followed by Calcofluor white staining. Microscopic pictures were taken with an Axiolab Microscope (Carl Zeiss) and AxioVisionRel4.6 software.

6.5.10 Adherence of *C. albicans* to TR146 cells

Adherence assay was performed as described (Murciano et al. 2012) with the following modifications. Monolayers of TR146 cells in 6-well plates were incubated with 10^2 *Candida* yeast cells for 2 hours at 37°C. The supernatant was then removed and distributed on YPD agar plates to determine the number of non-adherent fungal cells. After rinsing the 6-well plates with PBS the wells were overlaid with melted Wort agar at 40°C and incubated for 1 day at 37°C to count fungal colonies. Adherence was determined as (# of colonies grown on Wort agar) / (# of colonies grown on Wort agar) + (# of colonies grown from culture supernatant).

6.5.11 Cytokine secretion by TR146 cells

TR146 cells were grown to confluency in 96-well plates in Hams`s F12 nutrient mixture with 1% FCS and stimulated with $2x10^4$ *C. albicans* yeast cells per well for 24 hours. Human IL-6, IL-8 and IL-1α secretion by TR146 cells was quantified with the ELISA MAX Deluxe Set kits (Biolegend) according to manufacturer's instructions.

6.5.12 LDH release assay

The damage assay was performed as described (Wachtler et al. 2011a). Briefly, TR146 cells were grown to confluency in a 96-well plate in grown in Hams`s F12 nutrient mixture with 1% FCS and stimulated with $2x10^4$ *C. albicans* yeast cells per well for the indicated time period. Control wells were incubated with medium only or lyzed with 1% Triton-X-100 at the end of the incubation time to determine 100% damage. LDH release into the supernatant was quantified with the LDH cytotoxicity kit (Roche) according to the manufacturer's instructions.

6.5.13 RNA isolation and quantitative RT-PCR from *C. albicans*

Fungal cells were disrupted in Trizol reagent (Sigma) with glass beads (0.5 mm) in a FastPrep24 (MP Biomedicals) for 5 cycles of 1 min at 50Hz with cooling between cycles. RNA was isolated with Direct-zol RNA Mini Prep spin columns (Zymo research) and cDNA was generated by RevertAid reverse transcriptase (Thermo Scientific). Quantitative PCR was performed using Fast SYBR Green Master Mix and a FAST 3500 Genetic Analyzer (both Applied Biosystems). The primers were *Efb1* fwd 5'-CATTGATGGTACTACTGCCAC-3', *Efb1* rev 5'-TTTACCGGCTGGCAAGTCTT-3', *Kex1* fwd 5'-CTGATTCCGATTCCACCAGT-3', *Kex1 rev* 5'-ATGAGCGAGTGACATTGCT-3', *Kex2* fwd 5'-ATCGGCATCACAACAACAAA-3', *Kex2* rev 5'-GCTGATTGTTGTCCCTCCTC-3', *Hwp1* fwd 5'-CGGAATCTAGTGCTGTCGTCTCT-3', *Hwp1* rev 5'-TAGGAGCGACACTTGAGTAATTGG-3', *Ece1* fwd 5'-CGTTCCAGATGTTGGCCTTAATCT-3', *Ece1* rev 5'-CTGAGCCGGCATCTCTTTTAACTG-3', *Cph1* fwd 5'-GGTGGCGGCAGTGATAGTG-3', *Cph1* rev 5'-GTGTACTCCGGTGACGATTTTTC-3', *Als3* fwd 5'-TCTCGTCCTCATTACACCAACCAT-3', *Als3* rev 5'- GGGGATTGTAAAGTGGATTCTGTG-3', *Sap4* fwd 5'-

GCCGATGGTTCTGTTGCAC-3', *Sap4* rev 5'-GAGCCGCTATACTTGGCCTT-3', *Sap5* fwd 5'-TGGTTTTCAAAGCGGCGAAG -3', *Sap5* rev 5'-AGCATCAACATTTCGTCCCC-3', *Sap6* fwd 5'-CGTGGTGACAGAGGTGACTT-3', *Sap6* rev 5'-TGGTAGCTTCGTTGGCTTGG-3', *Sap9* fwd 5'-TTTCAGCGACACTTTGCAGC-3', *Sap9*rev 5'-TGTCGACTGTTCTGCTGGAG-3', *Efg1* fwd 5'-TCCCACCACATGTATCGACAA-3', *Efg1* rev 5'-GAAGTGCTCGAGGCGTTCA-3'.

6.5.14 Mice

Wildtype (WT) C57BL/6J mice were purchased from Janvier Elevage. *Il17ra*$^{-/-}$ and *Il17rc*$^{-/-}$ mice were obtained from Amgen (Thousand Oaks, CA) and bred at the Laboratory Animal Service Center (University of Zürich, Switzerland). All mice were on the C57BL/6 background, kept in (Solis, Filler 2012)specific pathogen-free conditions and used in sex- and age-matched groups at 6-12 weeks of age. All mouse experiments described in this study were conducted in strict accordance with the guidelines of the Swiss Animal Protection Law and were performed under protocols approved by the veterinary office of the Canton Zürich, Switzerland (license number 201/2012 and 183/2015). All efforts were made to minimize suffering and ensure the highest ethical and humane standards.

6.5.15 OPC infection model

Mice were infected sublingually with 2.5x10^6 cfu *C. albicans* yeast cells as described (Solis, Filler 2012) without immunosuppression. Mice were weighed daily. For determination of fungal burden, the tongue of euthanized animals was removed, homogenized in sterile 0.05% NP40 in H_2O for 3 minutes at 25 Hz using a Tissue Lyzer (Qiagen) and serial dilutions were plated on YPD agar containing 100 µg/ml Ampicillin.

6.5.16 Isolation of tongue cells

Mice were anaesthetized with a sublethal dose of Ketamine (100 mg/kg), Xylazin (20 mg/kg) and Acepromazin (2.9 mg/kg), and perfused by injection of PBS into the right heart ventricle prior to removing the tongue. Tongues were cut into fine pieces and digested with DNase I (200 µg/ml, Roche) and Collagenase IV (4.8 mg/ml, Invitrogen) in PBS at 37°C for 45-60 min. Single cell suspensions were

passed through a 70 µm strainer using ice-cold PBS supplemented with 1% FCS and 2 mM EDTA and analyzed by flow cytometry (see below).

6.5.17 Analysis of Th17 priming

Cervical lymph nodes were removed on day 7 post-infection and single cell suspensions were prepared by digested with DNase I (2.4 mg/ml, Roche) and Collagenase I (2.4 mg/ml, Invitrogen) in PBS for 15 min at 37°C. For inducing cytokine secretion by primed T cells, 10^6 cervical lymph node cells were re-stimulated for 6 hours with 10^5 DC^{1940} cells pulsed with $2.5x10^5$/ml heat-killed *C. albicans* or left unpulsed. Brefeldin A (10 µg/ml, AppliChem) was added for the last 5 hours. IL-17 production by $CD3^+$ $CD4^+$ T cells was analyzed by intracellular cytokine staining and flow cytometry (see below).

6.5.18 Flow cytometry

All antibodies were from BioLegend, if not stated otherwise. Single cell suspensions of tongues were stained in ice-cold PBS supplemented with 1% FCS, 5 mM EDTA, and 0.02% NaN_3 with LIVE/DEAD Fixable Near-IR Stain (Life Technologies), anti-CD45.2 (clone 104), anti-CD11b (clone M1/70), anti-Ly6C (clone AL-21, BD Biosciences) and anti-Ly6G (clone 1A8). For intracellular cytokine staining, T cells were first incubated in ice-cold PBS containing LIVE/DEAD Fixable Near-IR Stain, anti-CD4 (clone RM4-5) and anti-CD3ε (clone 145-2C11). After fixation and permeabilization using BD Cytofix/Cytoperm (BD Biosciences) the cells were then incubated in in Perm/Wash buffer (BD Biosciences) containing anti-IL-17A (clone TC11-18H10.1) and anti-IFNγ (clone XMG1.2) antibodies. Data were acquired on a FACS LSRII (BD Biosciences) or on a FACS Gallios (Becton Coulter) and analyzed with FlowJo software (Tristar). For all experiments, the data were pre-gated on live single cells.

6.5.19 RNA isolation and quantitative RT-PCR from mouse tissues

Isolation of total RNA from bulk tongues was carried out according to standard protocols using Trizol Reagent (Sigma). cDNA was generated by RevertAid reverse transcriptase (Thermo Scientific). Quantitative PCR was performed using SYBR Green (Roche) and a Rotor-Gene 3000 (Corbett Research) or a QuantStudio 7 Flex (LifeTechnologies). The primers were *Actb* fwd 5'-CCCTGAAGTACCCCATTGAAC-

3', *Actb* rev 5'-CTTTTCACGGTTGGCCTTAG-3'; *Cxcl2* fwd 5'-AGTGAACTGCGCTGTCAATGC-3', *Cxcl2* rev 5'-GCAAACTTTTTGACCGCCCT-3', *S100a9* fwd 5'-GTCCAGGTCCTCCATGATGT-3' and *S100a9* rev 5'-TCAGACAAATGGTGGAAGCA-3'; *Il17a* fwd 5'-GCTCCAGAAGGCCCTCAGA-3' and *Il17a* rev 5'-AGCTTTCCCTCCGCATTGA-3'; *Defb3* fwd 5'-GTCTCCACCTGCAGCTTTTAG-3' and *Defb3* rev 5'-ACTGCCAATCTG ACGAGTGTT-3'; *Il17f* fwd 5'- GAGGATAACACTGTGAGAGTTGAC-3' and *Il17f* rev 5'-GAGTTCATGGTGCTGTCTTCC-3'; *Il6* fwd 5'- GAGGATACCACTCCCAACAGACC-3' and *Il6* rev 5'-AAGTGCATCATCGTTGTTCATACA-3'; *Tnfa* fwd 5'- CATCTTCTCAAAATTCGAGTGACAA-3' and *Tnfa* rev 5'-TGGGAGTAGACAAGGTACAACCC-3'. All qPCR assays were performed in duplicates and the relative gene expression (rel. expr.) of each gene was determined after normalization with β-actin transcript levels.

6.5.20 Protein quantification by ELISA

Serum G-CSF and IL-6 were determined by sandwich ELISA according to standard protocols. The antibodies used were anti-G-CSF (clone 67604, R&D Systems) or anti-IL-6 (clone MP5-20F3, Biolegend) for coating and biotinylated polyclonal rabbit anti-G-CSF (Peprotech) or biotinylated anti-IL-6 (clone MP5-32C11, Biolegend) for detection, respectively. IL-17A and IL-17F cytokines were quantified by cytometric bead array (BD Biosciences) in tissue homogenates using the mouse IL-17F and IL.17A flex sets according to the manufacturer's instructions. For the preparation of tongue homogenates, the organ was cut in fine pieces and homogenated in 100 µl PBS supplemented with 1 x complete protease inhibitor cocktail (Roche) and 1% Triton-X-100 for 3 minutes at 25 Hz using a Tissue Lyzer (Qiagen). Data were aquired on a FACS Gallios (Becton Coulter) and analyzed with FloJo software (Tristar).

6.5.21 Histology and immunofluorescence

For histology, tissues were fixed in 4% PBS buffered paraformaldehyde overnight and embedded in paraffin, or embedded in O.C.T^{TM} compound (Tissue-Tek), snap-frozen in liquid nitrogen and stored at -20°C. Sagittal sections (9 µm) were stained with periodic-acid Shiff (PAS), counterstained with Haematoxylin and mounted with Pertex (Biosystems Switzerland) according to standard protocols. For

immunofluorescence staining, tissues were embedded in O.C.T™ compound (Tissue-Tek), snap-frozen in liquid nitrogen and stored at -20°C. Sagittal cryosections (9 µm), were fixed with acetone for 10 minutes at room temperature, stained with anti-IL-1α (clone ALF-161, BioXCell), anti-hamster-Cy3 (Jackson ImmunoResearch) and 4',6'-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) and mounted with Mowiol (VWR International AG). All slides were scanned with a NanoZoomer 2.0 HT (Hamamatsu Photonics K.K.) using a 20x objective, and analyzed using NDP.scan 2.5.88 software.

6.5.22 Transmission electron microscopy

Transmission electron tissue samples were fixed in 2.5% glutaraldehyde (EMS) buffered in 0.1 M Na-P-buffer overnight, washed x3 in 0.1 M buffer, post fixed in 1% osmium tetroxide (Sigma-Aldrich) and dehydrated in ascending concentrations of ethanol followed by propylenoxide and infiltration in 30% and 50% Epon (Sigma-Aldrich). At least three 0,9 µm toluidine blue stained semi-thin sections per localisation were produced. Representative areas were trimmed and subsequently 90 nm, lead citrate (Merck) and uranyl acetate (Merck) contrasted ultrathin sections were produced and viewed under Phillips CM10, operating with Gatan Orius Sc1000 (832) digital camera, Gatan Microscopical Suite, Digital Micrograph, Version 230.540.

6.5.23 Statistics

Statistical significance was determined by one-way ANOVA with Dunnett's or Sidak's multiple comparison test, or by Pearson correlation coefficient and linear regression as appropriate using GraphPad Prism (GraphPad Software) with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** $p < 0.0001$. Data displayed on a logarithmic scale were log-transformed before statistical analysis.

6.6 Acknowledgment

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Disclosures: The authors have no conflict of interest to declare.

6.7 **Supplementary Files**

Fig. S1

A

	CDC3		*EF3*		*HIS3*		*CA V*		*CA VII*	
SC5314	116	124	119	128	159	208	95	95	198	198
Cag	124	124	124	132	188	200	95	95	203	203
529 L	116	116	128	131	166	166	95	99	203	208
101	124	124	124	124	188	188	95	95	203	203
ATCC14053	116	124	128	128	159	179	95	95	198	317
UC820	112	116	124	128	146	146	95	95	198	213

B

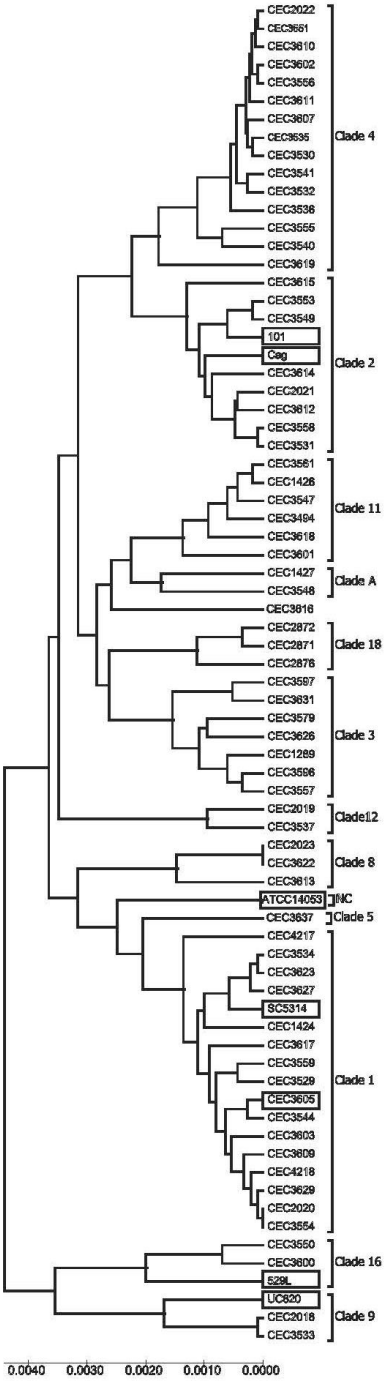


Figure S. 1 *C. albicans* isolates are genetically unrelated (A) Analysis of the microsatellite markers *Cdc3*, *Ef3*, *His3*, *CaV* and *CaVII* by PCR amplification followed by capillary electrophoresis. The alleles were assigned based on the detected fragment sizes in base pairs. (B) UPGMA dendrogram showing the genetic relatedness for the strains of *C. albicans* used in this study using MLST typing. Vertical bar to the right of each cluster indicate the clade designation. Scale indicates *P* distances. Numbers at nodal points indicate bootstrap values (%) for 1,000 replications. NC, not classified.

Fig. S2

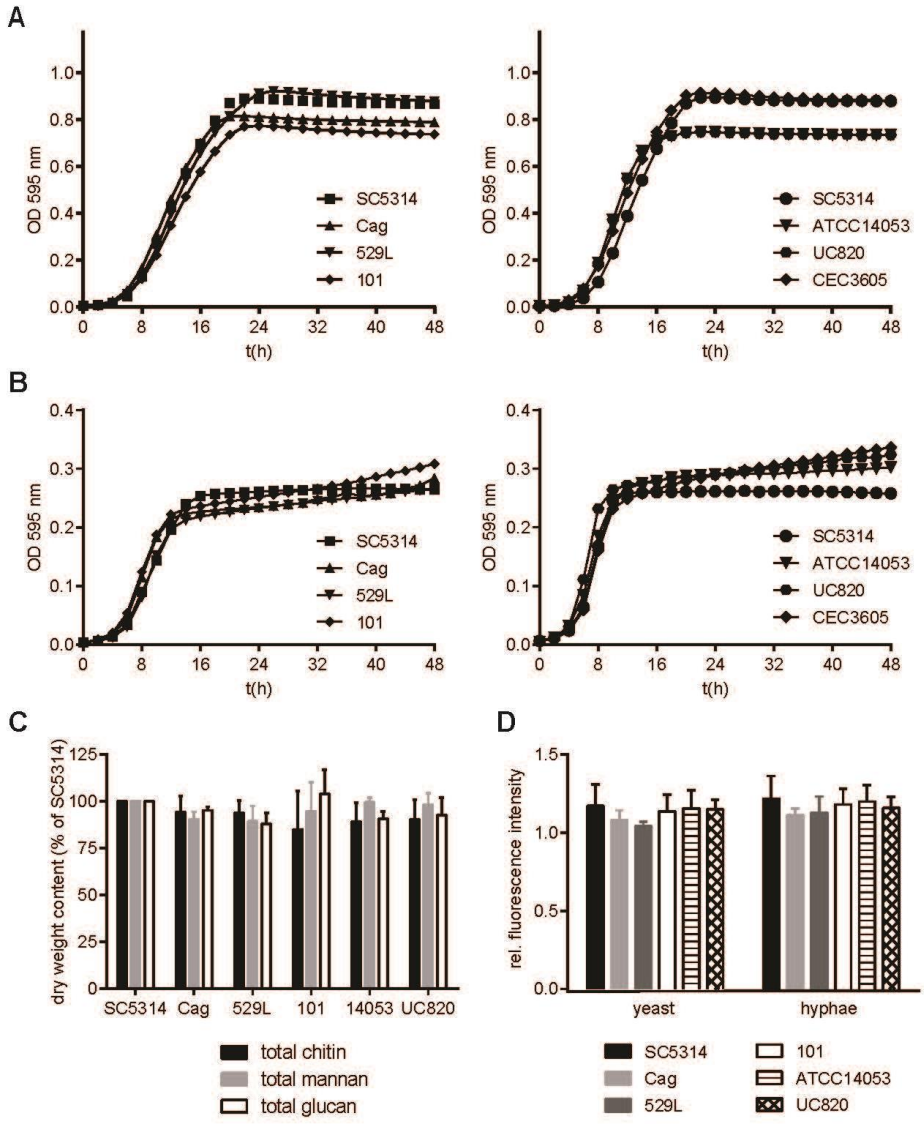


Figure S. 2. *C. albicans* isolates show normal growth and cell wall composition. (A – B) Growth of the indicated *C. albicans* isolates in YNB + 2% glucose medium (A) or F12 + 1% FCS medium (B) at 37°C was monitored over 48 hours using an automated plate reader. The OD_{595} was determined every 2 hours. (C) Total chitin, mannan and glucan contents of the indicated *C. albicans* isolates displayed as dry weight content relative to strain SC5314. (D) β-Glucan exposure of the indicated *C. albicans* isolates. For determining the relative fluorescence intensity for each isolate, the

fluorescence intensity of cells stained with the primary and secondary antibody was divided by the background signal from cells stained with the secondary antibody only.

Fig. S3

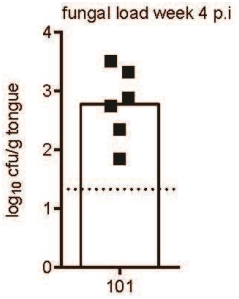
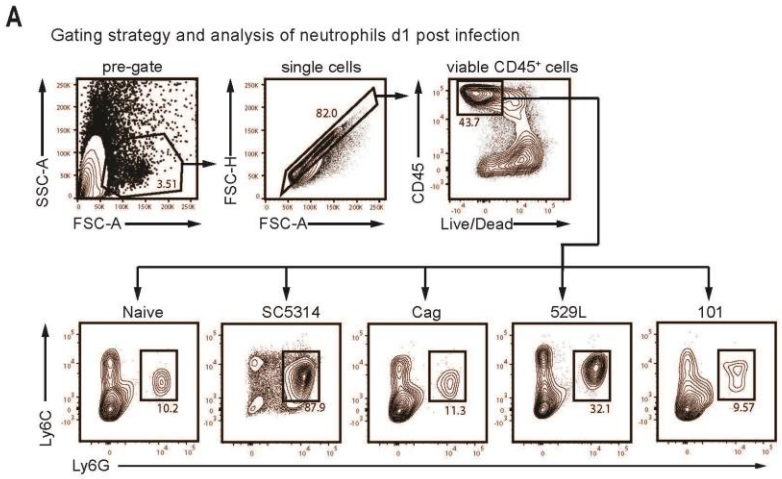


Figure S. 3 Strain 101 persists in the host for at least 4 weeks post-infection. The fungal burden in the tongue of WT mice 4 weeks post-infection with strain 101 is displayed as cfu/g tissue. Data are the pool of two independent experiments. Each symbol represents one mouse. The dotted line represents the detection limit.

Fig. S4



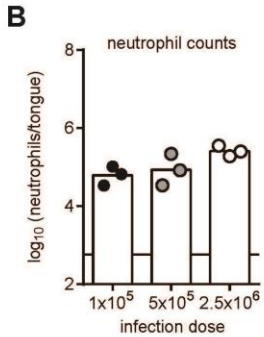


Figure S. 4 (A) Gating strategy for the quantification of viable $CD45^+$ $Ly6C^+$ $Ly6G^+$ neutrophils in the tongue tissue. (B) The neutrophil response to strain SC5314 is prominent irrespective of the infection dose. WT mice were infected with the indicated dose of strain SC5314 and $CD45^+$ $Ly6C^+$ $Ly6G^+$ neutrophils were quantified in the tongue on day 1 post-infection by flow cytometry. Each symbol represents one mouse. The mean of each group is indicated. A naïve group was included for control (depicted as a horizontal line). Differences between the infected groups were not significant if applying a one-way ANOVA and Dunnett`s multiple comparison test.

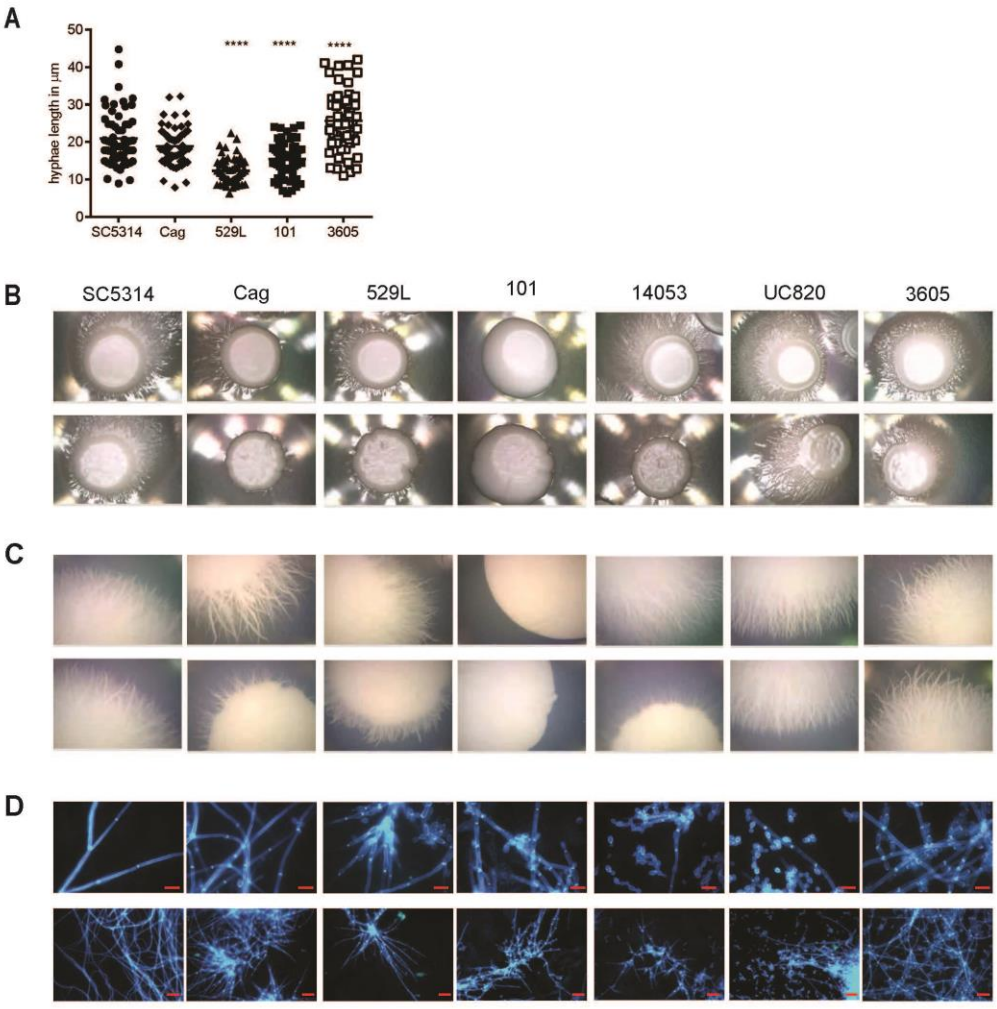


Figure S. 5 (A) *C. albicans* isolates were incubated on glass slides in FCS-containing medium and hyphal length was measured after 3 hours of incubation. Each symbol represents one measured hypha. The mean is also indicated. Data are a pool of two independent experiments. (B – C) Filamentation of *C. albicans* isolates was assessed on spider medium in top view (B) and by agar invasion (C). Representative images are shown for two concentrations of each isolate. (D) Fungal morphology after interaction with TR146 keratinocytes for 24 hours was determined after calcofluor white staining. Representative images at 40x (top row) and 100x magnification (bottom row) are shown for each isolate. Scale bars: 20 µm (40x) and 10 µm (100x).

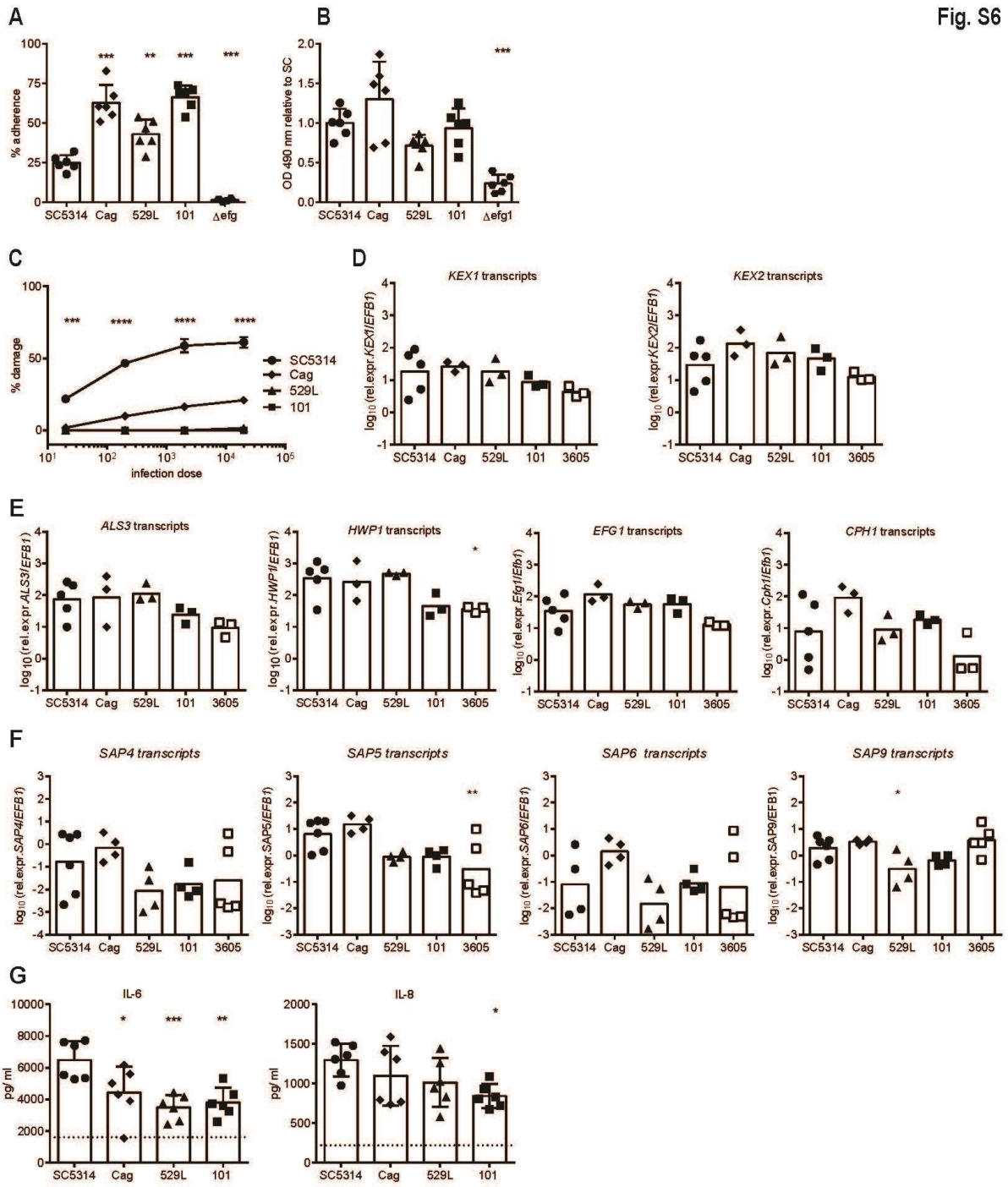


Figure S. 6 Interaction of *C. albicans* isolates with the oral keratinocyte cell line TR146. (A) Adherence of the indicated C. albicans isolates to TR146 cells was quantified after 2 hours of co-incubation. The efg1Δ/Δ strain was included as a negative control. (B) Biofilm formation of the indicated C. albicans isolates was quantified after 48 hours growth on medical silicon squares using the XTT assay. (C) LDH release by TR146 cells in response to gradual concentrations of C. albicans isolates measured after 24 hours of stimulation. (D – F) Expression of the indicated genes by C. albicans isolates upon contact with TR146 keratinocytes for 24 hours. (G) IL-6 and IL-8 secretion by TR146 cells in response to stimulation with the indicated C. albicans isolates for 24 hours. The dotted line represents the basal levels detected in the supernatant of unstimulated TR146 cells. In A, B and G, each symbol represents a separate well.

In D-F, data are the pool of 3 independent experiments. The mean and SD are indicated. Significant differences between each group and the strain SC5314 group are indicated.

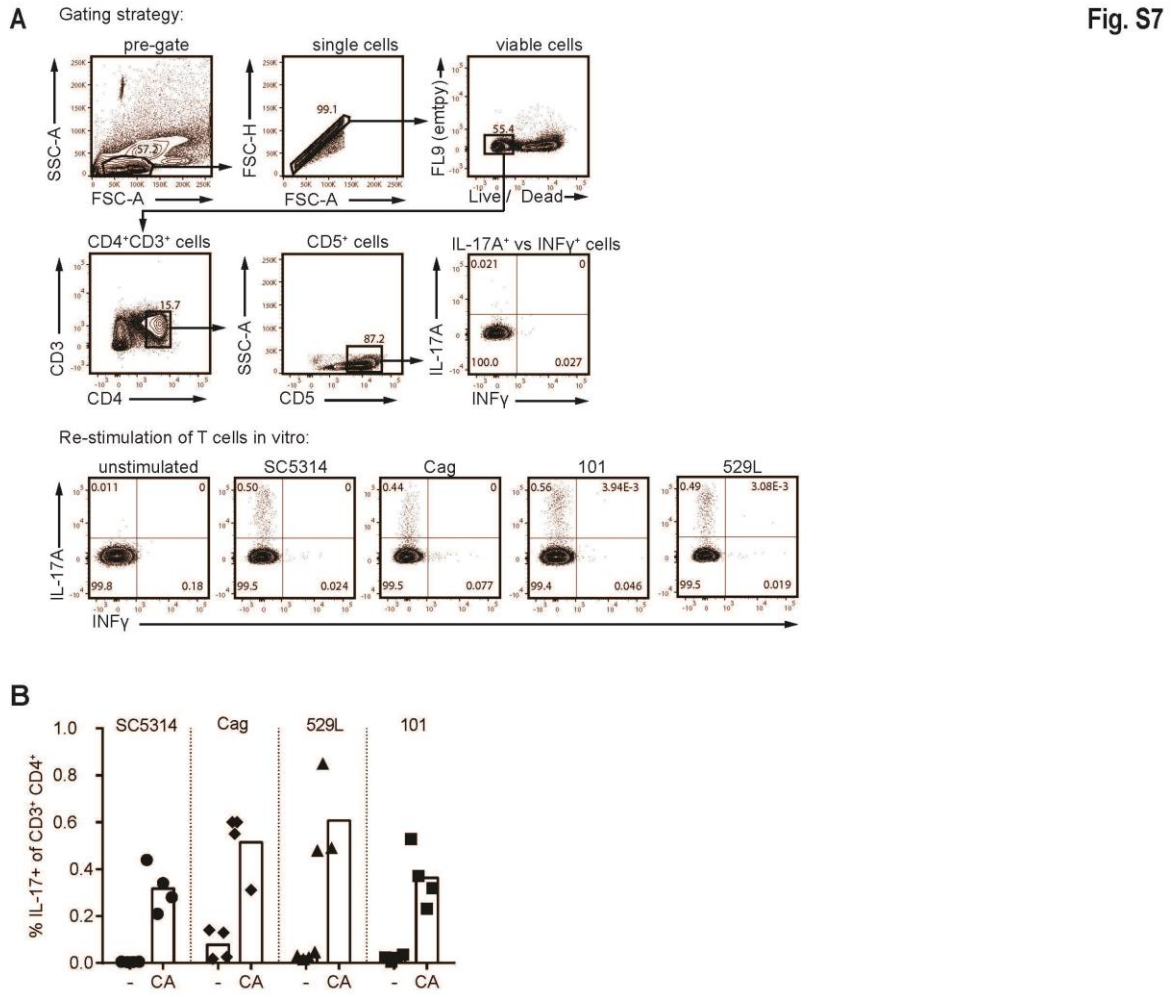


Figure S. 7 Adaptive immunity is induced normally by persistent fungal isolates despite the impaired induction of an acute response. WT mice were infected with the indicated isolates and IL-17A production by $CD3^+$ $CD4^+$ T cells was analyzed by flow cytometry on day 7 post-infection in the cervical lymph nodes after re-stimulation with *C. albicans*. (**A**) Gating strategy for single, viable, $CD4^+$ $CD3^+CD5^+IL17A^+$ cells. (**B**) Quantification of IL-17A$^+$ cells within $CD3^+$ $CD4^+$ T cells in response to infection with the indicated isolates. Each symbol represents one mouse. Data are representative of two independent experiments. Differences between *C. albicans* re-stimulated groups are not statistically significant if applying a one-way ANOVA and Dunnett`s multiple comparison test to SC5314.

Fig. S8

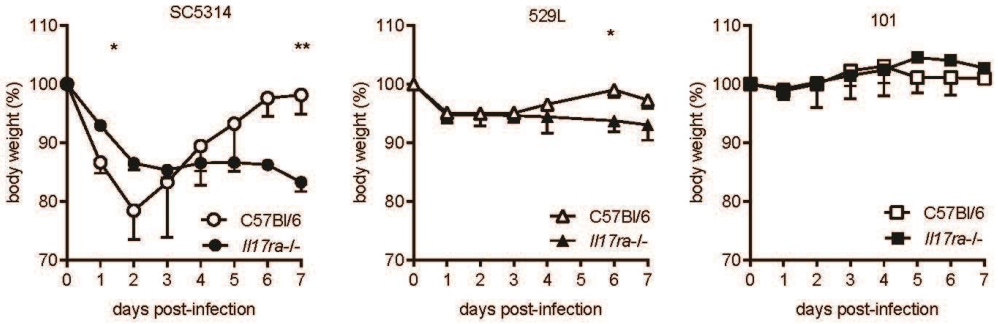


Figure S8. IL-17 signaling is essential for preventing fungal overgrowth irrespective of the degree of fungal pathogenicity.

Body weight of C57Bl/6 and *Il17ra*-/- mice in response to OPC infection with SC5314, 529L, and 101 over a period of 7 days. The mean + SD of three mice per group is shown.

7 Project III

Low-pathogenic strain of *C. albicans* evades immune attack in a co-infection with a highly pathogenic strain

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Author contributions: F.A.S and S.L.L. designed the experiments; F.A.S.performed the experiments; A.H. performed confocal fluorescence microscopy, F.A.S analyzed the data; F.A.S wrote the manuscript and S.L.L revised it. C.F.C. and S.L.L. supervised the project

7.1 Abstract

Candida albicans is an opportunistic fungal pathogen and resides usually on human mucosal surfaces without causing any symptoms. Under certain conditions it can also cause a variety of infections. The natural variability within *C. albicans* strains elicits a broad spectrum of host responses. In a previous study we reported that the highly pathogenic strain SC5314 induces a rapid and strong inflammatory response and is cleared rapidly. In contrast, the low-pathogenic strain 101 elicits only a diminished response and persists in the oral mucosa. By testing various strains, we found a strictly strain dependent correlation between the host inflammatory response and the subsequent clearing of the fungus.

Using an experimental model of oropharyngeal candidiasis, we investigated the cause-effect relationship between rapid immune induction and clearance of the fungus in a setting of co-infection with the low-pathogenic strain 101 and the high-pathogenic strain SC5314.

We could show that co-infection with a low- and high-pathogenic strain induces an acute inflammatory response. Surprisingly, we could demonstrate the persistence of the low-pathogenic strain 101 despite the induction of a strong inflammatory response by strain SC5314 in a co-infection setting. The resulting high immune response led to a selective clearing of the highly pathogenic strain SC5314. We show a close co-localization of both strains at the same areas of the tongue. Despite showing increased persistence in the oral mucosa, strain 101 did not show increased resistance to antimicrobial peptides or neutrophil killing.

This study emphasizes that persistent isolates colonize the oral mucosa in a manner distinct from high-pathogenic strains, whereby the exact mechanism remains to be elucidated. We could strengthen the notion that differences between *C. albicans* strains are relevant in infection.

7.2 Introduction

Over the last years, research on host-pathogen interactions gained broad interest. The majority of studies with experimental infections focused on the interaction with a single infectious agent, while mixed infections involving two or more strains of different genotypes were rarely noticed. Mixed infections include different species but also isolates belonging to the same species but to different populations or genotypes (Cox 2001) .

In mixed infections, pathogens influence each other: they can act synergistically, antagonistically or independently of each other (Mahuku et al. 1996). Infection with more than one genotype of the same pathogen occurs in many infectious diseases (Thompson 2000). In all cases, interspecies interactions increase the complexity of the disease (Lamichhane, Venturi 2015). Pathogen-pathogen interactions have been reported to increase disease severity in animals (Harms et al. 2001) in humans (Bosch et al. 2013) and, to a lesser extent, in plants (Lamichhane, Venturi 2015). In urinary tract infections, *Escherichia coli* enhances the adhesion of *Candida albicans* to the mucosa of the bladder (Levison, Pitsakis 1987). Moreover, patients colonized with *C. albicans* in the respiratory tract have a higher risk of ventilator-associated pneumonia caused by *Pseudomonas aeruginosa* (Azoulay et al. 2006).

Antagonism, on the other hand, is widely used in biological control of plant pathogens where one microbial antagonist is able to suppress a disease caused by another microbe (Pal, McSpadden

Gardener 2006). In plants, it has been shown that several fungal pathogens have the ability to induce resistance to high virulent pathotypes through low virulent isolates or saprophytes (Strömberg 1995; Martyn 1991). Infection of oilseed rape leaves with high and low virulent isolates of the fungal plant pathogen *Leptosphaeria maculans* leads to smaller lesions compared to an infection with the high virulent isolate alone. The isolate with low virulence can induce an acquired systemic resistance and coexist with the high virulent isolate in one lesion (Mahuku et al. 1996). Moreover, it has been shown that microorganisms inhabiting the same biological niche, thus competing for nutrients, often produce antimicrobial peptides (AMPs) to inhibit growth or kill competitors. In wine producing communities it has been found that *Saccharomyces cerevisiae* produces an AMP, active against other fungi including *C. albicans* (Branco et al. 2014). In human diseases, such as vaginal tract infections, it has been shown that lactic acid bacteria can compete with *C.albicans* for adhesion sites and secrete products which inhibit attachment (Boris, Barbés 2000).

C. albicans does not only interact with bacteria but also with other *Candida* species which are emerging as pathogenic agents of the oral cavity such as *Candida parapsilosis*, *Candida glabrata* or *Candida tropicalis* (Sardi et al. 2013). *C. albicans* and *C. glabrata* are often co-isolated from oral infections, which is an interesting clinical finding as *C. glabrata* alone only causes a negligible invasion and infection (Silva et al. 2011). For successful colonization of the oral mucosa, *C. glabrata* relies on co-infection with and adherence to *C. albicans* hyphae *(Tati et al. 2016)*.

The ubiquitous yeast *C. albicans* is one of the most important opportunistic human fungal pathogens (Zipfel et al. 2011; Duhring et al. 2015). As a commensal it colonizes the urogenital tract, the vaginal and gastrointestinal mucosae (Moyes, Naglik 2011) and can be detected in 30 to 50 % of the healthy adult population (Jacobsen et al. 2012). However, changes in the host can lead to the development of disease symptoms (Moyes, Naglik 2011) ranging from mild forms of oral and vulvovaginal candidiasis (Cheng et al. 2012) to severe life-threatening systemic infections (Bassetti et al. 2006). This shows that *C. albicans* can successfully colonize different niches of the body (Kumamoto, Vinces 2005). It is therefore not surprising to see high intraspecies genetic variations and a broad spectrum of attributes within isolates, such as variability in virulence in a murine model of intravenous challenge or general fitness (MacCallum et al. 2009; Hirakawa et al. 2015). Differences in the genotype between isolates include aneuploidy, chromosome truncations, translocations and loss of heterozygosity (Rustchenko 2007; Bennett et al. 2014). Intraspecies phenotypic differences are most likely a consequence of the differences in genotype. In a murine model of systemic infection, clinical isolates of *C. albicans* showed drastic variation in terms of virulence, which was also associated with differences in the growth rate (MacCallum et al. 2009). In addition, other *Candida* species, like *C. glabrata*, demonstrated strain-

dependent variation regarding their capacity to induce damage and cytokine production in epithelial cells (Li et al. 2007).

Our own work showed that natural *C. albicans* strains display a great spectrum in pathogenicity that results in different outcomes of the interaction with the host *in vivo* (Schönherr et al. 2017). Low-pathogenic strains, as 101, persisted in the mucosa and caused only delayed IL-17 induction, while highly pathogenic strains, as strain SC5314, elicited a rapid and strong immune induction and were quickly cleared from the oral cavity. In consequence to the differences in cytokine induction, neutrophil infiltration into the infected tissue was strictly strain-dependent. The rapid inflammatory response led to body weight loss on day 1-2 post-infection and subsequent recovery (Trautwein-Weidner et al. 2015b; Schönherr et al. 2017). Whereas strain 101 persisted in the oral mucosa for at least four weeks post-infection without causing body weight loss (Schönherr et al. 2017). The IL-17 pathway, although induced with delay by low-pathogenic strains, was shown to be essential for the growth control of all *C. albicans* strains. Key events for fungal clearance are IL-17-dependent antimicrobial mechanisms and IL-17 independent neutrophil infiltration and activation (Trautwein-Weidner et al. 2015b; Schönherr et al. 2017). The capacity of a strain to induce a rapid and strong inflammatory response in the host mucosa caused a fast clearance of the fungus from the host, while a diminished and delayed response led to persistence of strains in the host epithelium. (Schönherr et al. 2017)

The behavior of different strains has been mapped in experiments with single infections. Our previous data suggest that inflammatory response and infiltrating neutrophils are responsible for the fungal clearance. We aimed to further investigate this hypothesis. To address this, we made use of the well-established model of oropharyngeal candidiasis (OPC) and performed co-infection experiments with the highly pathogenic strain SC5314, which induces a rapid neutrophil response, and the low-pathogenic strain 101, for which we did not observe a significant neutrophil response. We postulated different outcomes: (I) both strains might act together in a synergistic way and induce a higher inflammatory response leading to the clearance of both strains, (II) strain SC5314 would act as an antagonist and repeal the (unknown) mechanism of the low-pathogenic strain to persist in the host, (III) low-pathogenic strain 101 suppresses the inflammatory response induced by the other strain leading to persistence of both strains in the mucosa, (IV) the two disparate strains might act independent without influencing each other in the host.

Using an experimental OPC model, this study shows that co-infection with a low and high-pathogenic strain induces an acute inflammatory response. Consequently, persistence of strain 101 cannot be explained by suppression of the immune response. Surprisingly, the overall high immune response led to a selective clearing of the high-pathogenic strain. The hypothesis of an independent action of the

strains seems to be most likely. This study emphasizes that persistent isolates colonize the oral mucosa in a different manner than highly pathogenic strains. The induced inflammatory response and the resulting clearance of the fungus is strictly strain dependent.

7.3 Results

7.3.1 Co-infection of mice with two *C. albicans* strains with divergent pathogenicity induces an acute inflammatory response

To investigate the interaction of two different *C. albicans* strains with opposite pathogenicity in the host, we performed co-infection experiments. To discriminate the two strains *in vivo*, we made use of fluorescently labelled variants of both *C. albicans* strains.

When used for single infection, the highly pathogenic strain SC5314 induced a rapid and strong inflammatory response, including massive neutrophil infiltration and high induction of IL-17 and antimicrobial peptides on day 1 post-infection, as reported previously (Conti et al. 2009; Trautwein-Weidner et al. 2015b; Gladiator et al. 2013; Schönherr et al. 2017). In contrast, the low-pathogenic strain 101 elicited a diminished IL-17 and antimicrobial response and only few neutrophils infiltrated to the site of infection at this time point (Schönherr et al. 2017). We examined the fungal burden and initial host response in co-infection with a 1:1 mixture of SC5314-GFP and 101-mCherry (**Figure 14**). As a control, we used 1:1 mixtures of either 101-GFP and 101-mCherry or SC5314-GFP and SC5314-dTomato. Quantification of the overall fungal load showed a slightly reduced fungal burden on day one post-infection in mice infected with the combination of high- and low-pathogenic strains compared to single-strain-infections. However, the ratio of the two admixed strains was stable. Next, we investigated the host response elicited by the combination of the two disparate strains. Analysis of single cell suspensions from co- and single-strain-infected tongues by flow cytometry revealed an equally strong neutrophil response in co-infected mice as in mice infected with strain SC5314 alone, while no neutrophil response was induced by strain 101, as previously reported (Schönherr et al. 2017). Similarly, the coinfection of mice with the two strains did not affect the induction of IL-17 cytokines and IL-17 target genes, such as *S100A9*, which was observed in response to infection with strain SC5314 alone.

Together, these results indicated that the presence of strain 101 did not impair/suppress the host response induced by strain SC5314. Also, the co-infection did not elicit an enhanced inflammatory response compared to single infection, thus excluding synergism between the two strains.

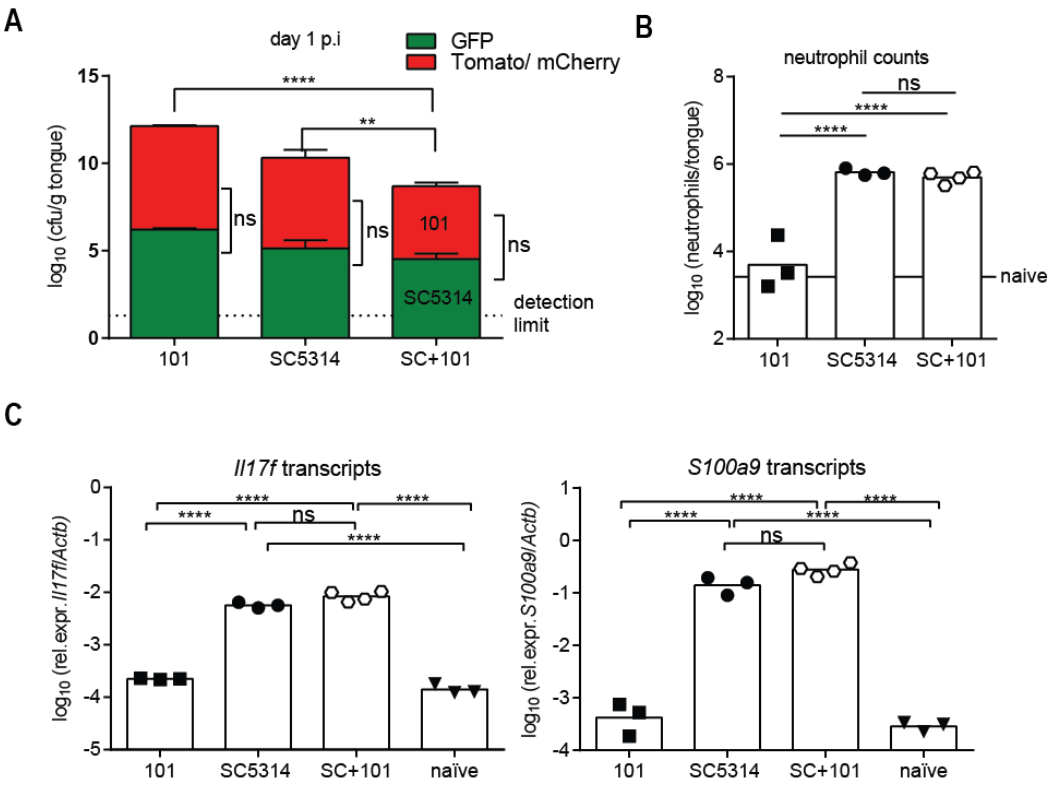

Figure 14 Mice infected with a 1:1 mixture of *C. albicans* strains 101 and SC5314 show decreased fungal burden but no diminished immune response. Wildtype mice were sublingually infected with a 1:1 Mix of SC5314-GFP and 101-mCherry101-GFP and 101-mCherry or SC5314-GFP and SC5314-dTomato or SC5314, and analyzed on day one. (A) Fungal burden in the infected tongue displayed as colony forming units (cfu) per g tongue. Data are the mean of two independent (mix) or one (single-strain- infections) experiments with three mice per group. The dotted line represent the detection limit. (B) $CD11b^+$ $Ly6C^+$ $Ly6G^+$ neutrophils in the tongue of infected and naïve control mice were quantified by flow cytometry. The solid line represents the mean neutrophil counts of three naïve mice. (C) *Il17f*and *S100a9* mRNA expression in the tongue tissue of infected and naïve control mice was assessed by qRT-PCR. In B and C, each symbol represents one mouse. Statistical analysis was performed using one-way ANOVA and Dunnett´s multiple comparison test. Significant differences between each group and the strain SC5314 single-strain-infected group are indicated.

7.3.2 Only the low-pathogenic strain persists in the oral mucosa during co-infection with a high-pathogenic strain

As expected from the strong innate immune response to a co-infection with the low- and high-pathogenic strains SC5314 and 101, the mice displayed a strong drop in body weight (**Figure 15A**). The drop in weight loss in the co-infected group was slightly less pronounced compared to single-strain infection with SC5314. We wondered what the impact of this response was on the persistence of the strains in the host.

Next, we assessed the fungal burden in single-strain and co-infected animals on day 7 post-infection (**Figure 15B**). The fungus persisted in the co-infected and 101 single-strain infected animals, while SC5314 single-strain infected animals cleared the fungus. The use of fluorescence labelled strains enabled us to discriminate between the high and low-pathogenic strains. In co-infected tongues only the low-pathogenic 101-mCherry could be detected, whereas SC5314-GFP was completely cleared. SC5314 single-strain infected mice almost completely cleared the fungus, whereas strain 101 persisted in the host in a roughly 1:1 ration of mCherry and GFP labelled strains.

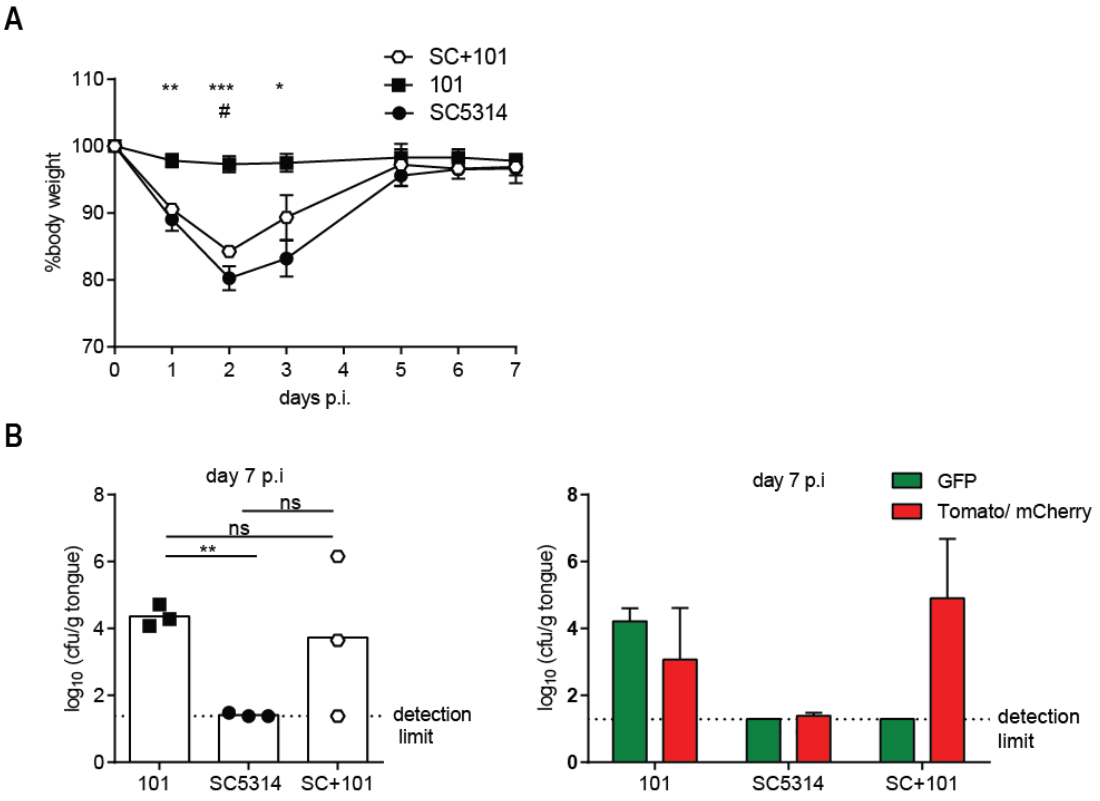

Figure 15 Selective clearance of the high-pathogenic isolate by day seven post-infection. Wildtype mice were sublingually infected with a 1:1 mixture of 101-GFP and 101-mCherry, SC5314-GFP and SC5314-Tomato or SC5314-GFP and 101-mCherry. (A) Body weight of infected mice over a period of 7 days. The mean + SD of 3 mice per group is shown. Statistical analysis was performed using one-way ANOVA and Dunnett´s multiple comparison test. Significant differences between each of the single-strain-infected groups and the co-infection group are indicated by asterisks or hashes, respectively. (B) Fungal burden in the infected tongue on day 7 post-infection displayed as cfu per g tongue. Each bar is the mean + SD of 3 mice per group. The dotted line represents the detection limit. No statistical differences were obtained by using one-way ANOVA and Dunnett´s multiple comparison test.

A strong expression of *Il17* and high numbers of infiltrating neutrophils, as a consequence of infection with strain SC5314, were associated with fungal clearance from the host (Schönherr et al. 2017).

Surprisingly, this did not occur for strain 101 in the setting of co-infection and the low-pathogenic strain could persist in the oral mucosa.

7.3.3 Altering the ratio of high-pathogenic strain in co-infection does not change the outcome of infection

We wondered whether the rapid and acute response elicited by high-pathogenic strain SC5314 might have concealed a possible immunosuppression by strain 101 in the co-infection setting. Therefore, we examined if a change in the ratio of strain 101 and strain SC5314 in the co-infection setting changed the outcome of the infection. We performed OPC infection with a 25 times and 5 times reduced amount of strain SC5314 in single and co-infections with a constant number of 2.5 $^{x}10^{6}$cells of strain 101.The fungal burden in co-infected tongues was significantly higher for all ratios tested if compared to single infections with strain SC5314 (white bars) on day 7 post-infection (**Figure 16A**). The fungal load of single-strain infected tongues with strain 101 (black bar to the very right) was comparable to co-infections with 25 times, 5 times reduced or normal infection dose as tested before. In addition, neutrophil infiltration was not affected by the change of the ratio in co-infections. Neutrophil numbers of SC5314 single and co-infected mice did not reveal any differences at all ratios tested. All tested mixes with strain SC5314 led to a significantly stronger infiltration of neutrophils compared to single infection with strain 101. These results suggest that even at low infection doses, strain SC5314 was still highly pathogenic. Lowering the infection dose by a factor of 25 reduced the amount of infiltrating neutrophils only very minutely (**Figure 16B** white bars).

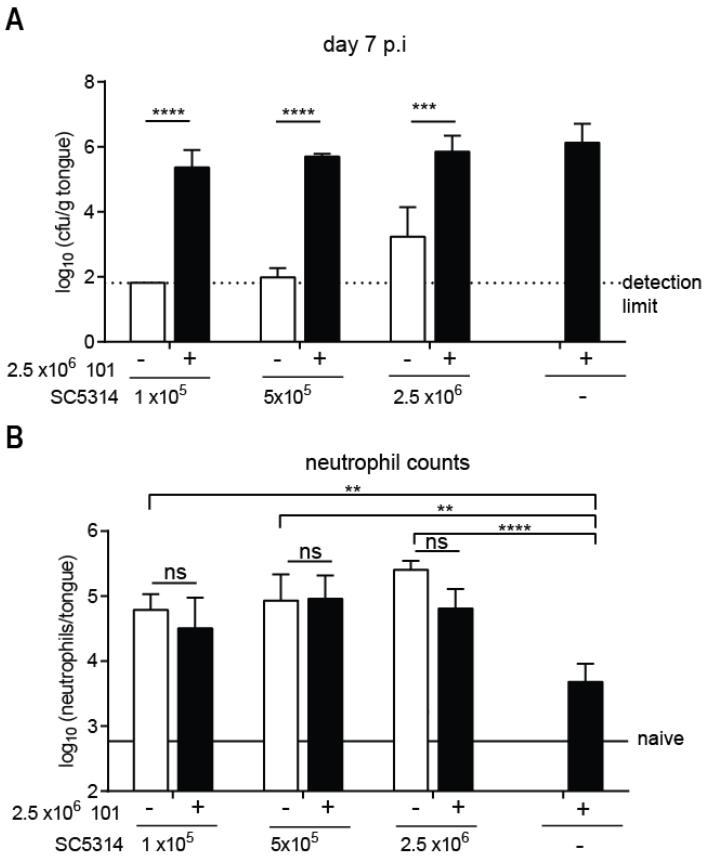

Figure 16 The behavior of the highly pathogenic strain SC5314 in the host was not dose-dependent. Wildtype mice were sublingually infected with the indicated dose of SC5314-GFP alone or in combination with $2.5x10^6$ 101-mCherry cells. (A) Fungal burden in the infected tongue on day 7 post-infection displayed as cfu per g tongue. The dotted line represents the detection limit. (B) $CD11b^+$ $Ly6C^+$ $Ly6G^+$ neutrophils in the tongue of infected and naïve mice were quantified by flow cytometry on day 1 post-infection. The horizontal line represents the mean neutrophil counts of three naïve mice. The mean + SD of 3 mice per group is shown. Statistical analysis was performed using one-way ANOVA and Dunnett´s multiple comparison test. Significant differences between each group are indicated.

7.3.4 Both strains colonize the same regions of the tongue

The persistence of strain 101 in co-infected mice suggests that this strain may selectively resist or evade the strong inflammatory response in the oral mucosa. We thus hypothesized that the two strains may segregate spatially in the infected tongue and that strain 101 may not get in close contact with the inflammatory mediators that are induced by the other strain. To test this hypothesis we examined the localization of the fluorescently-labelled strains by fluorescence microscopy analyzing co-infected tongues in top-view. To avoid quenching of the fluorescent reporters, we analyzed unfixed, whole tongues infected with SC5314-GFP and 101-mCherry (**Figure 17**). Fluorescence and confocal microscopy showed close co-localization of both strains in most of the infected regions. These findings therefore make the spatial segregation of the two strains as an explanation for the selective survival

of strain 101 in the co-infection setting rather unlikely. Lingual papillae and *Candida* hyphae of both strains were visible despite the strong auto-fluorescence of the epithelial cells.

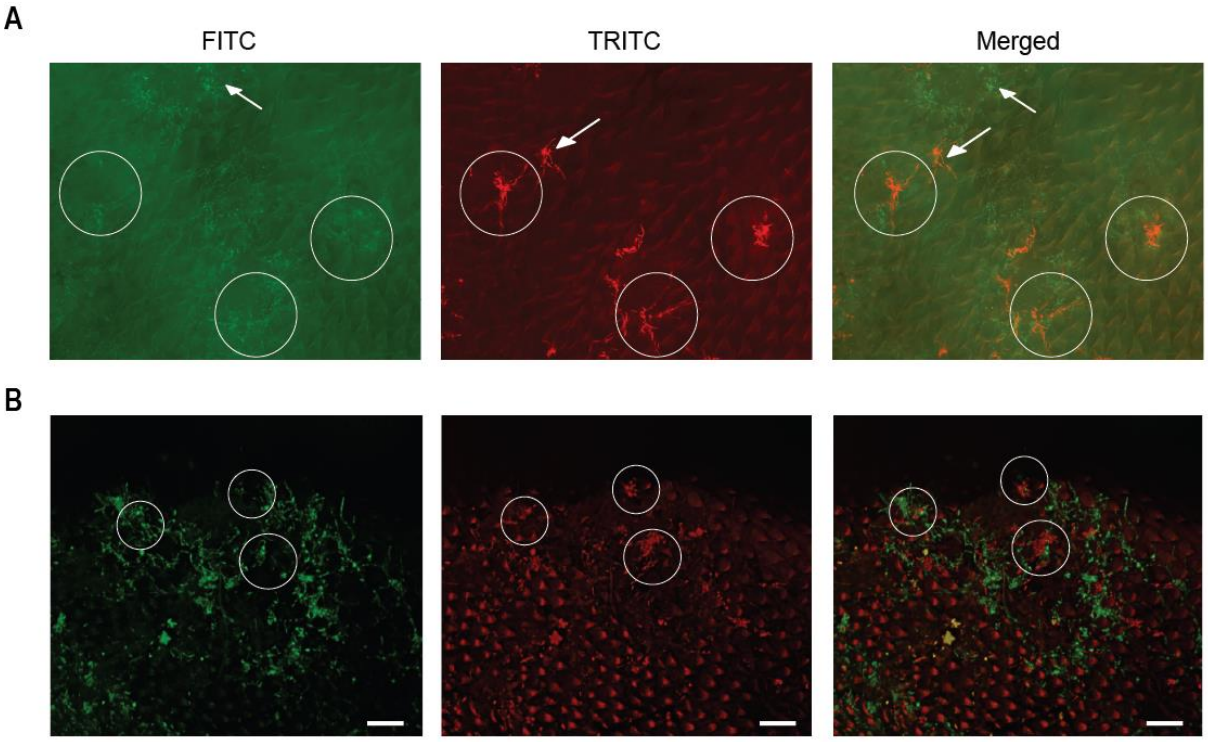

Figure 17 High and low-pathogenic isolates reside at the same sites in the tongue. Representative images of unstained, unfixed whole tongues co-infected with SC5314-GFP and 101-mCherry by fluorescence microscope (A) or fluorescent confocal microscope (B) in top view on day 1 post-infection. White circles highlight areas with both strains. The white arrows indicated areas with only one strain.

7.3.5 The low-pathogenic strain 101 does not display an enhanced resistance to host defense mechanisms

We did not observe an obvious spatial segregation, therefore strain 101 is likely exposed closely to the local response induced by strain SC5314. We further investigated if strain 101 displayed an enhanced resistance to inflammatory and antimicrobial response (**Figure 18**).

Neutrophils are the central weapon of the innate immune system. Neutrophils infiltrate directly to the site of infection (Lukacs et al. 1999) and prevent fungal dissemination and overgrowth (Trautwein-Weidner et al. 2015b). We therefore tested the resistance of strain 101 to neutrophils (**Figure 18A**). For this, we isolated neutrophils from the bone-marrow of naïve mice and compared the killing rate after incubation with mouse-serum opsonized yeast and hyphae of strain 101 and strain SC5314. The low-pathogenic strain 101 was killed with a higher efficiency by neutrophils than strain SC5314.

Antimicrobial peptides are a second very important branch of the host response to control *C. albicans* at the mucosal barrier (Dale, Fredericks 2005). We therefore assessed resistance of *C. albicans* strains 101 and SC5314 to human β-defensin-3 (hBD3), Histatin 5 (HST 5) and Cathelicidin (LL-37) *in vitro* (**Figure 18B**). Both strains displayed the same sensitivity to hBD3 and HST 5, with a MIC of 500 ug/ml and > 500 ug/ml respectively. LL-37 did not affect the viability of either strain. Together, these results suggested that strain 101 was not more resistant to two key antifungal host mechanisms in the oral mucosa and that the increased persistence of this strain in the oral mucosa can thus not be explained by any of the raised hypotheses.

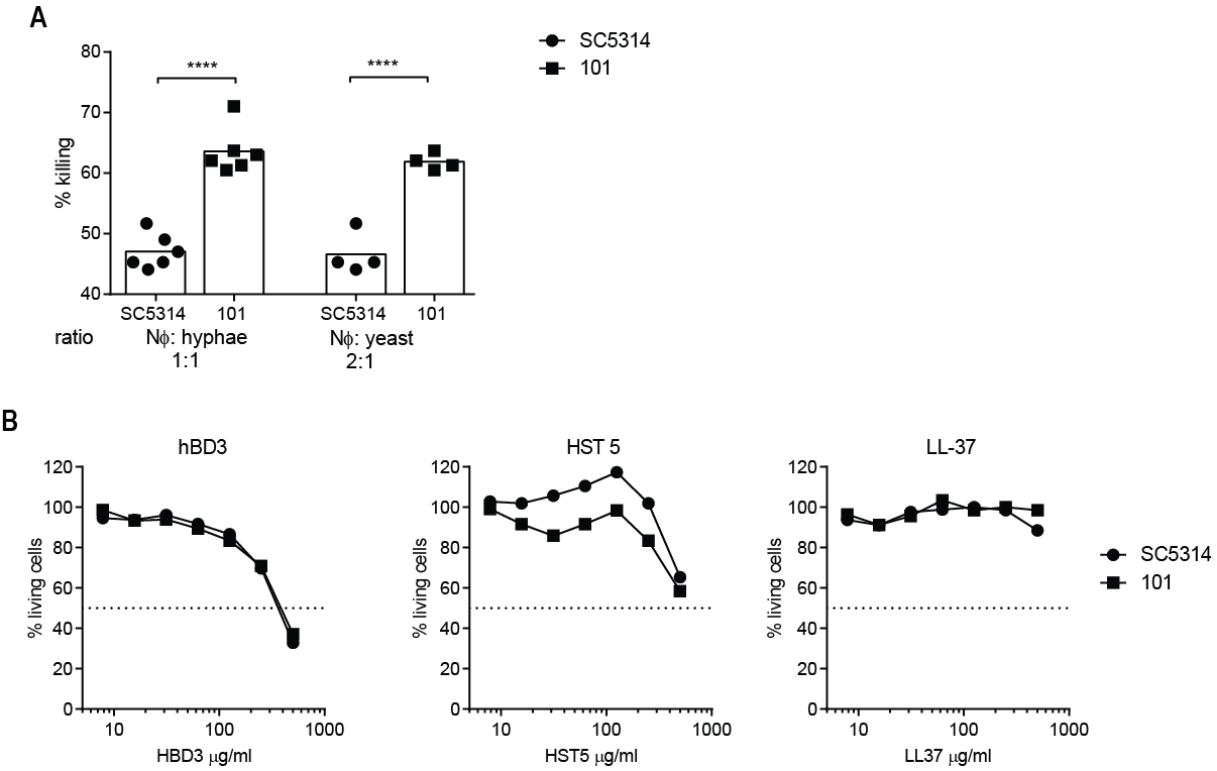

Figure 18 Low-pathogenic strain 101 is more sensitive to neutrophil killing but showed no enhanced susceptibility to antimicrobial peptides (A) Neutrophils from the bone marrow of naïve mice where co-incubated with opsonized hyphae and yeast cells and the percentage of killed *Candida* cells was assessed by alamar blue (B) The Antimicrobial peptides human Beta-Defensin-3 (Hbd3), Histatin 5 (Hst5), Cathelicidin (LL-37) were incubated at indicated concentrations with *Candida* strain SC5314 and 101 and cell numbers were assessed. (C) TR146 cells were stimulated with 101, SC5314 and a mix of both isolates. LDH release was determined after 24 hours.

While it is known that low-pathogenic strain 101 persists in the host, we could show that the same outcome occurs during an acute initial immune response induced by a high-pathogenic strain in a co-infection (Schönherr et al. 2017).

7.4 Discussion

As an opportunistic yeast, *C. albicans* is usually harmless on human mucosal surface but under specific circumstances it can also cause a variety of infections. The natural variability within *C. albicans* isolates contributes to the decision between commensalism and pathogenicity and thereby to the outcome of the interaction with the host. We used co-infection of a low- and high- pathogenic strain in the well-established OPC model to investigate the cause-effect relationship between rapid immune induction and clearance of the fungus. We could demonstrate the persistence of the low-pathogenic strain 101 despite the induction of a strong inflammatory response by strain SC5314 in a co-infection. The two strains occupied the same areas of the tongue. Strain 101 did not show an increased resistance to neutrophil killing or antimicrobial peptides *in vitro*. All these findings indicated that both strains act independent from each other, rather than synergistic or antagonistic, in a co-infection setting. Our study strengthens the notion that differences between *C. albicans* strains are considerable and relevant in infection.

Since van Leuwenhook in the 1600s, it is known that microbes are part of complex communities (Gest 2004). Co-infections might occur from a reinfection before the previous one is cleared (Read, Taylor 2001). Co-infection of a host with a high- and a low-pathogenic strain of the same species, as we have examined in this study, may result in an enhanced response through synergism between the two strains, a reduced response through suppression by one strain or it may stay unaltered. In addition, mixed infections can impact the dynamics of the infecting pathogen populations in different ways: the burden of both agents may increase, one or both may be suppressed or one may have a higher burden while the other decreases (Cox 2001). The outcome of the infection is determined by the variability in pathogenicity of *C.albicans* strains.

During OPC, *C. albicans* strains 101 and SC5314 can colonize the tongue tissue in a comparable manner when analyzed in single-infections, whereas co-infection with the two led to slightly reduced fungal load on day one post-infection. Interaction between the strains due to competition for nutrients might have affected the colonization efficiency or limit replication of one strain (Thomas et al. 2003).

The massive infiltration of neutrophils and strong expression of IL-17and S100A9 triggered by strain SC5314 were not altered in the co-infection setting. Infections with strain 101 were accompanied with a diminished neutrophil infiltration .(Schönherr et al. 2017). In contrast, the high and rapid immune response induced by strain SC5314 also occurred in the co-infection. Indicating that strain 101 did not suppress the response induced by strain SC5314.

Of importance, the high and acute attack of the immune system did not affect the persistence of the low-pathogenic strain 101. The fungal burden on day seven post-infection was similar to single infections with strain 101. The use of fluorescence labelled strains in the co-infection revealed that only strain 101 remained in the host, while strain SC5314 was cleared. This suggests that a synergistic interaction between the two strains can be ruled out, as both strains would have been cleared or it would have affected the capacity of strain 101 to persist in the host.

C. albicans has developed various efficient strategies to interfere, suppress and evade immune attacks from the host (Zipfel et al. 2011). The co-infection did not raise a higher immune response compared to the single infection with the high-pathogenic strain. It is however possible that the acute response caused by strain SC5314 might have concealed a possible immunosuppression by strain 101. To reduce the efficiency of strain SC5314, mixed infection with 4% of the initial dose was tested. Surprisingly lower dose of strain SC5314 did not change the infection outcome characterized by high neutrophil infiltrates and clearance of strain SC5314. With these findings, immunosuppressive actions from strain 101 could be excluded as both strains would have persisted in the oral mucosa in this case.

There are different possible explanations for how strain 101 evades the immune system (Collette, Lorenz 2011; Zipfel et al. 2011) but strain 101 seems neither to impair nor to suppress the immune response. Avoidance of killing by infiltrating neutrophils could occur due to distinct localizations of the two strains on the tongue tissue. Although fluorescence microscopy revealed colonization in the same areas of the tongue if looked in two-dimensional views, it is conceivable that strain 101 and SC5314 reside at different depths of the squamous epithelium cells or display an enhanced intrinsic resistance to inflammatory cells. Even though it was shown neutrophils infiltrate in the epithelial layer where *C. albicans* resides (Trautwein-Weidner et al. 2015b). Invasion depth to an early point of infection revealed that strain SC5314 reaches into deeper epithelial layers, whereby strain 101 resides in the upper layers (Schönherr et al. 2017). Intrinsic enhanced resistance to inflammatory responses was tested *in vitro* by neutrophil and antimicrobial peptide killing, but only differences in neutrophil killing between strains were detected, with strain 101 being killed with a higher efficiency. Putative differences to inflammatory cells (e.g. macrophages), which are not neutrophils are also possible. The mechanism through which strain 101 avoided being attacked and cleared by neutrophils as well as infection dynamics elicited by strain 101 remain unclear . Further research is needed to reveal/clarify the mechanism behind the strain specific evasion of strain 101 from the immune system. Competitive interactions between the strains may occur and consequently affect the dynamics within the host. In addition, the host seems to be able to discriminate between the low-pathogenic strain 101 and the high-pathogenic strain SC5314.

It is established that strain 101 can coexist with the host without causing symptoms of disease, but additional studies are needed to investigate how strain 101 is able to persist (Schönherr et al. 2017). The fungus might only reside in the mucosal tissue, mainly in the stratum corneum, without proliferating and growing. The fungal burden between two and four weeks post-infection remains stable (Schönherr et al. 2017). Therefore an equilibrium of growth and killing by the host immune system is also possible.

To conclude, our study using a low and a highly pathogenic strain of *C. albicans* in the OPC infection model, allowed us to gain further insights on the interplay between the host immune response and highly diverse *C. albicans* strains in single-strain and co-infection. The reason for the persistence of strain 101 remained unclear and requires further investigation. Yet, we could exclude some putative scenarios for selective persistence of strain 101, while others remain to be tested. Our study highlights strain specific differences in host- pathogen interaction which have to be considered in *in vivo* fungal infection.

7.5 Material and Methods

7.5.1 Fungal strains and media

The *C. albicans* blood strain SC5314 (Gillum et al. 1984b) and the oral strain 101 (Schönherr et al. 2017) were previously reported. SC5314-GFP was reported before (Igyarto et al. 2011). SC5314-CAF2-yTomato was constructed analog to CAF2-mCherry (Brothers et al. 2011) but in SC5314 inserting dTomato ura3Δ::imm434/URA3 P_{ADH1}-dTomato_$NAT_R$and a kind gift from R.Wheeler. 101-GFP, 101-mCherry express GFP or mCherry under the control of the ADH promoter. The construct is integrated at the ADHI locus with SAT1 as resistance marker. Both strains were a kind gift from D. Sanglard. All isolates were maintained on Sabouraud or Yeast Peptone Dextrose (YPD) agar plates for short term and in YPD supplemented with 20% glycerol at -80°C for long term storage. For experiments, *C. albicans* was inoculated from a preculture at an OD_{595} = 0.1 and grown in YPD medium at 30°C and 180 rpm for 15-18 hours, or 12 hours if used for the damage and antimicrobial peptide assays. For hyphae induction, *Candida* cells were adjusted to the indicated cell concentration and incubated for 3 hours at 37°C in PBS supplemented with 5% FCS. Opsonization was done for 30 minutes on ice in PBS supplemented with 20% or 10 % mouse serum for yeast or hyphal cells, respectively.

7.5.2 Ethics statement

All mouse experiments described in this study were conducted in accordance with the guidelines of the Swiss Animal Protection Law and were performed under protocols approved by the Veterinary office of the Canton Zürich, Switzerland (license number 201/2012 and 183/2015). All efforts were made to minimize suffering and ensure the highest ethical and humane standards.

7.5.3 OPC infection model

Mice were infected sublingually with $2.5x10^6$ *C. albicans* yeast cells as described (Solis, Filler 2012) without immunosuppression. Mice were weighed daily. For determination of fungal burden, the tongue of euthanized animals was removed, homogenized in sterile 0.05% NP40 in H_2O for 3 minutes at 25 Hz using a Tissue Lyzer (Qiagen) and serial dilutions were plated on YPD agar containing 100 µg/ml Ampicillin.

7.5.4 Isolation of tongue cells

Mice were anaesthetized with a sublethal dose of Ketamine (100 mg/kg), Xylazin (20 mg/kg) and Acepromazin (2.9 mg/kg), and perfused by injection of PBS into the right heart ventricle prior to removing the tongue. Tongues were cut into fine pieces and digested with DNase I (200 µg/ml, Roche) and Collagenase IV (4.8 mg/ml, Invitrogen) in PBS at 37°C for 45-60 min. Single cell suspensions were passed through a 70 µm strainer using ice-cold PBS supplemented with 1% FCS and 2 mM EDTA and analyzed by flow cytometry (see below).

7.5.5 Flow cytometry

All antibodies were from BioLegend, if not stated otherwise. Single cell suspensions of tongues were stained in ice-cold PBS supplemented with 1% FCS, 5 mM EDTA, and 0.02% NaN_3 with LIVE/DEAD Fixable Near-IR Stain (Life Technologies), anti-CD45.2 (clone 104), anti-CD11b (clone M1/70), anti-Ly6C (clone AL-21, BD Biosciences) and anti-Ly6G (clone 1A8). Data were acquired on a FACS LSRII (BD Biosciences) or on a FACS Gallios (Becton Coulter) and analyzed with FlowJo software (Tristar). For all experiments, the data were pre-gated on live single cells.

7.5.6 RNA isolation and quantitative RT-PCR from mouse tissues

Isolation of total RNA from bulk tongues was carried out according to standard protocols using Trizol Reagent (Sigma). cDNA was generated by RevertAid reverse transcriptase (Thermo Scientific). Quantitative PCR was performed using SYBR Green (Roche) and a Rotor-Gene 3000 (Corbett Research) or a QuantStudio 7 Flex (LifeTechnologies). The primers were *Actb* fwd 5'-CCCTGAAGTACCCCATTGAAC-3', *Actb* rev 5'-CTTTTCACGGTTGGCCTTAG-3', *S100a9* fwd 5'-GTCCAGGTCCTCCATGATGT-3' and *S100a9*

rev 5'-TCAGACAAATGGTGGAAGCA-3'; *Il17f* fwd 5'-GAGGATAACACTGTGAGAGTTGAC -3' and *Il17f* rev 5'-GAGTTCATGGTGCTGTCTTCC -3'. All qPCR assays were performed in duplicates and the relative gene expression (rel. expr.) of each gene was determined after normalization with β-actin transcript levels.

7.5.7 Neutrophil killing assay

Neutrophils were purified from the bone marrow of naïve C57Bl/6 mice by density gradient purification (Swamydas et al. 2015). To test neutrophil-killing activity, the indicated number of *C. albicans* yeast or hyphae were exposed to $5x10^3$ neutrophils for three hours before the neutrophils were lysed with water supplemented with 0.02% Triton-X-100 and the number of surviving yeast/hyphae cells was assessed with alamar blue. A standard curve was generated with serial dilutions of yeast or hyphae cells. Killing activity is expressed as percent of cells surviving in the presence of neutrophils compared to the conditions without neutrophils. *Candida* yeast or hyphal cells were opsonized with fresh mouse serum, as described below.

7.5.8 Antimicrobial peptide assay

Antimicrobial peptides LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), Hst 5 (DSHAKRHHGYKRKFHEKHHSHRGY) and hBD-3 (GIINTLQKYYCRVRGGRCAVSCLPKEEQIGKCSTRGRKCCRRKK) were obtained from Synpeptide (China). *C. albicans* cells from a 12 hours overnight culture were harvested, washed twice in PBS and incubated for two hours at 30°C. Afterwards, cells were adjusted to $1x10^6$cells/ml, 100 µl were transferred to every well of a 96-well plate and the plate was centrifuged. The supernatant was removed and the cell pellet was resuspended in 25 µl working solution of the indicated concentration of the antimicrobial peptide or PBS as untreated control. After incubation for 3.5 hours at 37°C, the wells were washed with PBS and the supernatant removed. Percentage of surviving cells was assessed by alamar blue. A standard curve was generated with serial dilutions of *Candida* cells.

7.5.9 Statistics

Statistical significance was determined by one-way ANOVA and Dunnett´s multiple comparison test using GraphPad Prism (GraphPad Software) with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Data displayed on a logarithmic scale were log-transformed before statistical analysis.

8 General Discussion

8.1 Prevalence of *Candida* colonization

Candida spp. are important fungal pathogens. The clinically most relevant species are *C. albicans, C. tropicalis, C. glabrata, C. guilliermondii, C. krusei, C. lusitaniae, C. parapsilosis,* and *C. stellatoidea* (Odds 1988). Transmission is commonly vertically, although cases of horizontal transmission have been reported, predominantly in nosocomial settings (Bliss et al. 2008). The increasing use of immunosuppressive therapies and broad-spectrum antibiotics predispose individuals to opportunistic fungal infections. Under these special conditions the host is unable to control overgrowth of the colonizing fungus and to prevent its switch from a commensal to pathogenic state (Singh 2001). Colonization with *Candida* spp. in the oral cavity or digestive tract are a known risk factor for oral and disseminated candidiasis (Fanello et al. 2006; Charles et al. 2005; Gammelsrud et al. 2011; Nucci, M. and Anaissie, E. 2001).

Reported prevalence of *Candida* spp. ranges between 26% and 71% in healthy children from Turkey and the United Kingdom, respectively (Martin, Wilkinson 1983; Kadir et al. 2005). We aimed to investigate the prevalence of *C. albicans* colonization in a defined population (healthy children) in a distinct geographical area (southern Switzerland), which is of high medical importance.

It was the first study to investigate the colonization rates in 130 healthy children in a local population in southern Switzerland. We could show that with 19.2%, the colonization rate with *Candida* spp. was lower in this local population than the majority of reported numbers from other geographical regions. Only a few studies, for example from India and Turkey, detected a lower prevalence of *Candida* spp. (8% and 5%) in groups of healthy children between six and nine years (Smiline et al. 2012; Akdeniz et al. 2002). In contrast to our study, only the oral cavity was tested while we examined the perianal region, skin and oral cavity.

In agreement with the literature we detected *C. albicans* (11.5%), as the most prevalent species among all *Candida* spp. (Jarvis 1993; Abi-Said et al. 1997; Schuman et al. 1998; Bassetti et al. 2006). Nevertheless, *non-albicans* species are on the raise, most possibly due to the increased use of fluconazole and other azole drugs (Nguyen et al. 1996). *C. glabrata* and *C. krusei* are naturally multi-drug resistant and consequently represent a severe health hazard (Pfaller et al. 2008). We had no cases of *C. glabrata* or *C. tropicalis* colonization in our cohort, and only one child was colonized with *C. krusei*

in the tested population in southern Switzerland. Other identified species, with less prevalence, were *C. guilliermondii*, *C. tropicalis* and *C. pelliculosa.*

We showed that the prevalence and distribution of *Candida* spp. is lower in our investigated cohort. Suggesting that the geographical area may impact the prevalence and distribution. Raising the importance in knowledge of local epidemiology. Further well defined studies are needed to elucidate the impact of the geographical region on prevalence and a possible influence on cases of oral candidiasis in the same population. *C. albicans* isolates are commonly associated with the host over long periods and transmissions usually occur during birth from the mother to the child (Bougnoux et al. 2006; Bliss et al. 2008). Epidemiological studies on the prevalence of vulvovaginal and oral colonization in healthy individuals in southern Switzerland would proof or dispel the theory of an overall low prevalence of *Candida* spp. in the investigated region.

8.2 Genetic variability among *C. albicans* isolates

Microsatellites are hypervariable and frequent markers for differentiation of clinical isolates. We analyzed 88 isolates from healthy children and adults and diseased individuals by MLMT. Tested individuals were colonized by clonal populations. Across all individuals, the isolates revealed a high genetic diversity, independently from isolation of healthy or diseased individuals.

From the three tested loci, *CDC3*, *EF3* and *HIS3* by microsatellite analysis, several allelic combinations presented homologous fragment sizes in both alleles. It remains unclear if this is the result of the same length of the amplified fragments or due to loss of heterozygosity (Sampaio et al. 2005). Chromosomal alterations, like loss of heterozygosity, are widely reported and can be associated with the loss of chromosomal parts or the entire chromosome (Rustchenko et al. 1994; Rustchenko-Bulgac 1991; Tavanti et al. 2004).

Three sequence types (ST) showed homologous allelic combinations in all three tested loci. The isolates could be haploid, homozygous diploid, might have loss of heterozygosity events at the three loci or the same fragment size on both alleles (Sampaio et al. 2005; Hickman et al. 2013). Genomic rearrangements can occur as an adaptive response to environmental stress conditions (Selmecki et al. 2010). Loss of heterozygosity can be induced by the exposure to fluconazole, other stressors, like heat shock or changed carbon source (Hilton et al. 1985; Perepnikhatka et al. 1999; Rustchenko et al. 1994). *C. albicans* populations within an individual are mostly clonal and adaptation to changing conditions through sexual reproduction is rather unlikely. An observed adaptation is the phenotype switching to

the grey morphology, in which mating is more efficiently compared to the white morphology (Tao et al. 2014). The above discussed genomic rearrangements could confer a selective advantage in a host environment. On transcriptional level, it has already been demonstrated that tristable phenotypic switching between white, gray and opaque cells with distinct genetic expression profiles play a role in adaptation to the host (Tao et al. 2014). To further address this in more details, long-term studies are needed to clarify if isolates adapt on a genetic level to host stresses or if even strain replacement occurs.

We detected a high genetic diversity, based on the fragment size of the Microsatellites, in *C. albicans* isolates from healthy children in southern Switzerland. Furthermore, we analyzed isolates from healthy children and adults and diseased individuals. The 88 obtained isolates clustered in 59 ST´s. The obtained diversity in the diseased population is comparable to the variation found in healthy individuals. Analysis by MLST would provide further insight to affiliation to one of 18 described phylogenetic clades (Odds et al. 2007).

It was proposed that distinct ST´s or clonal complexes have a higher pathogenicity or host adaptation (Dalle et al. 2003). In agreement with other studies, with partly different typing methods, we showed a similar distribution of isolates from healthy and diseased individuals across different clonal complexes (Bougnoux et al. 2002; Dalle et al. 2003), thus disproving the original idea.

In project II we could correlate induced immune response *in vivo* and pathogenicity, defined by damage induction in epithelial monolayers *in vitro*. Two Isolates (SC5314, ATCC14053) described in project II belong to the same clonal complex 1, but to different ST and showed massive differences in their pathogenicity. The isolates 529L, 101 and Cag could not be assigned to any clonal complex and are singletons. Isolate UC820 was grouped in clonal complex 2 and 3605 was not tested by MLMT. With this approach we tested a large collection of isolates (irrespective of the collection in project I) from superficial and invasive origin. Isolates described as high, mid and low-pathogenic did not segregate according to the origin. It remains questionable if phenotypic differences can reflect the clustering in clonal complexes by MLMT or clades by MLST. Deep sequencing might reveal more insight into natural genetic differences underlying the phenotypic differences.

8.3 Fungal determinants define the balance between commensalism and pathogenicity

A broad diversity exists among natural isolates of *C. albicans*, both from a phylogenomic as well as a phenotypic perspective. However, little attention has been paid so far to the contribution of the intraspecies variation of *C. albicans* to the decision between health and disease. We assessed the host response to natural *C. albicans* isolates in the murine OPC model, where the impact of fungal differences can be studied in a *Candida*-naïve environment, which is complex but uniform. We demonstrated a broad spectrum of temporally and qualitatively distinct immune responses to *C. albicans* isolates.

8.3.1 *C. albicans* strains induce distinct host immune responses *in vivo*

Highly pathogenic isolates led to a strong and rapid induction of inflammation, which was correlated with fungal clearance. Low-pathogenic isolates persisted for at least 4 weeks in the host, which was linked to a low induction of inflammation. Even a delayed induction of the IL-17 pathway and an antimicrobial response did not result in fungal clearance. Surprisingly, these drastic differences in the initial acute host response to distinct *C. albicans* isolates did not translate into differences in the adaptive immune response. Irrespectively of the pathogenicity and consequent capacity to initiate an innate response, the subsequently induced Th17 response was comparable. The quality of the T cell response to highly and low-pathogenic isolates has to be further investigated.

8.3.2 Selective persistence of low-pathogenic strain 101 in co-infection

Induction of a rapid and acute inflammatory response appeared to be decisive for persistence or fungal clearance in single-strain infections. We challenged this hypothesis in a setting of co-infection with two fungal isolates with opposing behavior, namely the high-pathogenic strain SC5314 and the low-pathogenic strain 101. During a co-infection, strain SC5314 elicited a strong immune response as during single-strain-infection, but this did not affect the persistence of strain 101 in the same host. Consequently, we could rule out that strain 101 actively suppressed the induction of a host response, which would have also influenced clearance of SC5314 during co-infection.

The mechanism of immune evasion of strain 101, as an example for a persisting, low-pathogenic strains, remains to be further investigated. We could show co-localization of both strains on the tongue when looking vertically/in top-view, but we can not exclude a separation in different epithelial cell layers of the mucosal tissue. We speculate that the two distinct isolates may actually segregate with respect of their invasion depth, as we observed in single-strain-infections, where low-pathogenic

strains were restricted to the stratum corneum and the high-pathogenic strains invaded the stratum granulosum and spinosum. A possible approach to answer this question could be microdermabrasion of the stratum corneum of the tongue prior to infection with a low- or high-pathogenic strain or co-infection of the two, to generate a situation in which both strains reach the stratum granulosum/spinosum. In experiments with skin, the stratum corneum largely reformed within the first 24 hours after microdermabrasion (Andrews et al. 2011). This would allow us to modify the initial contact site of the fungus with the host and target *C. albicans* low-pathogenic isolates to a different epithelial layer, which likely determines the outcome of the disease. Effector molecules might not reach the stratum corneum, in which persistent strains reside, infection of the stratum spinosum can proof or dispel this hypothesis.

We observed drastic differences in invasion depth of distinct *C. albicans* isolates at an early time point of infection, before neutrophil infiltration can keep the invading fungus under control. Activation of c-Fos and the subsequent proinflammatory cytokine response in epithelial cells to *C. albicans* is dose-dependent (Moyes et al. 2010). During exposure of epithelial cells with a low fungal burden, the oral epithelial cells may remain "silent", whereas an infection with damage-inducing hyphae and/or a higher fungal burden can induce a strong proinflammatory response in the epithelium (Moyes et al. 2010; Jacobsen et al. 2012). However, our findings could not be correlated with differences in the morphology or fungal load of the isolates. We detected only small differences in hyphae formation with two triggers for hyphae formation, 37°C and serum. Differences were more pronounced after contact with epithelial cells, whereas the proportion of cells forming pseudohyphae or hyphae, the hyphal length and the degree of branching highly varied.

The fungal burden at the onset of infection was comparable among all isolates, although we assessed the fungal load in the whole tongue and we can not distinguish between cells which adhere at the surface and invaded cells. This could be further investigated in a simplified system with a 3-dimensional model of epithelial cells. Staining with fluorescent conjugate of Concanavalin A stains only non-invaded fungal elements and would give insight on the proportion of invaded and surface-attached fungal cells (Wachtler et al. 2011b). Not only the fungal burden at the onset of infection, but also growth in the host tissue might give fitness advantages and impacts the outcome of the infection. It is unclear if isolates actively grow or only reside in the host tissue after OPC infection. In order to determine the behaviour in the host, a reporter model with photoconvertible *C. albicans* strains could be used (SC5314-mKIKGR, 101-mKIKGR) (Muller et al. 2013). The photoconvertible fluorescent protein mKikumeGR expression was introduced in the pathogen under the promoter of the alcohol dehydrogenase in order to quantify its growth. The green fluorescent protein in the pathogen converts

to red when illuminated with violet light. As the pathogen grows and proliferates, new green proteins are produced and the growth can be quantified.

8.3.3 Morphology does not correlate with virulence and induction of epithelial immune response

Filamentation per se does not appear to be sufficient for pathogenicity and damage induction (Noble et al. 2010; Tang et al. 2016; Moyes et al. 2016). The impact of the different *C. albicans* morphologies on the induction of the host immune response is still not well understood (Jacobsen et al. 2012). It was proposed that the yeast-to hyphae transition is one of the key virulence factors and non-filamentous strains show an attenuated virulence (Lo et al. 1997b; Thompson et al. 2011; Zheng, Wang 2004). Infection with a yeast-locked strain in immunocompetent mice led to no severe infection, whereas the same strain induced a high killing rate in immunocompromised mice (Saville et al. 2008).

Hyphae are considered to be the pathogenic phenotype, since they can penetrate tissue and cause damage (Jacobsen et al. 2012). However, a mutant (ece1Δ/Δ), which formed normal hyphae is unable to induce a damage response and thereby was attenuated in its capacity to trigger an inflammatory response in the host when compared to the parental strain (Moyes et al. 2016). Proteolytic cleavage of the Ece1 protein leads to the release of a peptide toxin called Candidalysin which was described to be required and sufficient for induction of epithelial damage by strain SC5314 (Moyes et al. 2016). However, in our study we could not correlate filamentation, *ECE1* expression and damage induction in epithelial cells *in vitro.* In support of our findings, a previous study by Noble et al. also uncoupled infectivity and morphogenesis. These authors showed that infectivity attenuated mutants revealed a normal morphological transition (Noble et al. 2010). More refined analysis of filamentation under conditions more closely linked to the situation *in vivo* are needed. We assessed filamentation on the surface of the tongue, but investigation of invaded fungal cells remains open. We could not correlate morphological differences with the varying degrees of damage induction. It is most likely that damage, at least partly, is induced by secreted factors. Supernatants from infected epithelial cell culture were sufficient to cause damage (data not shown). Proteomics of the supernatant and the secretome in the saliva of infected mice, might give more insight into extracellular factors causing damage and influencing the host response. Extracellular vesicles (EV) containing RNA, polysaccharides, pigments, proteins and lipids have been found in culture supernatants from fungal pathogens, like *C. albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Malassezia sympodialis* (Joffe et al. 2016; Vargas et al. 2015). Proteomic analysis revealed proteins related to pathogenesis and stress response, as a result EVs might interfere with the host response (Vargas et al. 2015).

The origin of the isolates we tested (mucosal surfaces or blood cultures) did not segregate according to differences in pathogenicity. This is in contrast to a study, suggesting tissue-specific virulence of *C. albicans* isolates (Taylor et al. 2000). Herein, mucosal isolates were virulent in the vaginitis infection model, whereas most of these isolates were less virulent in the systemic model (Taylor et al. 2000).

We tested several know virulence attributes to find a possible explanation of the observed differences in pathogenicity between isolates seen i*n vivo.* An important virulence attribute of *C. albicans* is fungal adhesion and invasion to host cells (Wachtler et al. 2011b). Proteins of the Als family (e.g. Als3) play a role in cell-surface, cell-cell contacts and invasion (Sheppard et al. 2004). All tested isolates included in our study were able to adhere to epithelial cells and to form biofilms, even though at a variable degree. The differences in adherence and biofilm formation did, however, not correlate with *ALS3* expression levels, which were comparable in all isolates. In accordance with other studies, *C. albicans* isolates showed intraspecies variation in their ability to adhere to different substrates and to form biofilm (da Costa et al. 2012; Li et al. 2003).

A possible explanation for differences in pathogenicity between *C. albicans* isolates could also be at the level of fungal recognition by innate immune cells. An example for this is provided by a study which showed different recognition by innate immune cells between two unrelated isolates in mice lacking the β-1,3-glucan receptor Dectin-, which was based on differences in the composition of their cell walls (Marakalala et al. 2013). However, we did not observe differences in the cell wall composition and degree of β-glucan exposure among the tested isolates.

We revealed striking differences in the induction of damage in epithelial cells and were able to link this to the release of IL-1α *in vitro.* Furthermore, IL-1α induction could also be shown *in vivo* in response to two extreme isolates exemplified by strains SC5314 and 101. IL-1 signaling is linked to the regulation of neutrophil recruitment in response to *C. albicans* (Altmeier et al. 2016b; Tomalka et al. 2015; Tomalka et al. 2011b). Our data demonstrated that the differential induction of an inflammatory response, like infiltrating neutrophils, correlated closely with the induction of damage in keratinocytes *in vitro*. This knowledge provided us with the advantage to investigate a larger collection of isolates as it would be possible with mouse experiments in vivo. We performed a preliminary study with 76 isolates from superficial or invasive origin. The clustering in high, mid and low damage induction capacity served as a surrogate for determining the degree of pathogenicity, which correlated with the induction of inflammation in the host tissue *in vivo.* The underlying impact of the *C. albicans* isolate genome on the host response still remains unclear, but the high degree of the possible sequence variation between individual isolates is a big challenge (Hirakawa et al. 2015; Ford et al. 2015). Variation in the genome will be difficult to translate to phenotypic differences, due to several

regulatory levels in between. Proteomics of the secretome in the saliva of infected mice (see above) and RNA-seq from the fungus in the infected tongue, might reveal more information.

8.3.4 Fungal adaptation to host stresses

Gene expression is dependent on environmental stimuli and provides a fast adaptation to changing conditions and niches. Expression of many virulence factors is regulated at the level of transcription in response to host encounter. We measured the expression levels of known virulence factors of *C. albicans* in contact with epithelial cell culture but did not find significant differences, apart from Ece1 expression (as discussed above). A central role in the pathogenicity of C. albicans is extracellular proteolytic activity with several functions in the infective process (Naglik, JR et al. 2003a). SAP transcripts in human saliva or vaginal swabs are more frequently detected in oral and vaginal infection compared to asymptomatic carriage (Naglik et al. 2003). Analysis of secreted proteins and transcripts of *C. albicans* in the saliva of OPC infected mice might provide better insights in host adaptation. *C.albicans* is, independent of being in a commensal or pathogenic state, exposed to different stressors of the host such as changes in temperature, nutrient availability, pH, osmolarity or attack by neutrophils and antimicrobial peptides (Wilson et al. 2009). On the other hand, the fungus seems to have an adaptation which is predictive, preparing for subsequent events. For example, genes which mediate oxidative stress response are already expressed before the actual host response occurs (Jacobsen et al. 2012). Adaptation to host stress might vary among isolates, which can be further investigated by RNA-seq in the setting of OPC infection in mice. The low-pathogenic strain 101 appear to be more susceptible to neutrophil killing *in vitro* compared to the highly pathogenic strain SC5314. Nevertheless, in the setting of co-infection 101 resides in the epithelium despite the infiltrating neutrophils, suggesting a possible higher resistance to oxidative stress by reactive oxygen species.

Our study clearly showed that not only host immune pathways are activated differently to contribute to antifungal defense and immunity. Natural variations between the fungal isolates result in selective activation of different defense mechanisms and influence the outcome of the fungal-host interaction with impact on fungal clearance or possible persistence, independent of the host immune state.

8.4 The hallmark cytokine IL17 plays a non-redundant role in immune response to *C. albicans* irrespective of the pathogenicity

Dysregulation of IL-17 is associated with psoriasis, rheumatoid and psoriatic arthritis and the immunopathology causing these autoinflammatory diseases (Gregersen, Olsson 2009; Di Cesare et al.

2009) and most research on IL-17 has been devoted to understanding the host-adversive effects of the cytokine. IL-17 is an important therapeutic target, either be blocking IL-17A directly or its receptor, and available on the market with spectacular results in remission of psoriasis, rheumatoid and psoriatic arthritis (Lonnberg et al. 2014).. However, from an evolutionary perspective, IL-17 has a host-beneficial role. More specifically, IL-17 is a key regulator of the mucosal immunity and protective host response to fungal infections. Superficial candidiasis is occasionally observed as a side effect of IL-17 targeting, underlining the protective role of IL-17 in antifungal immunity (Welch et al. 2015). The non-redundant role of IL-17 has been identified in individuals with genetic defects involved in the differentiation of Th17 cells or in IL17 signaling that display an increased susceptibility to mucocutaneous candidiasis (Cypowyi et al. 2012).

In several mouse models, infections with *Histoplasma capsulatum*, *Aspergillus fumigatus* and *C. albicans*, the absence of IL-17 blunted the clearance of the fungus (Deepe, Gibbons 2009; Werner et al. 2009; Gladiator et al. 2013; Trautwein-Weidner et al. 2015b; Kashem et al. 2015a).

A robust innate immune response is needed to protect the host from superficial and systemic candidiasis (Jacobsen et al. 2012). Likewise, it has been shown that depletion of critical components of the host immune response, such as neutrophils or IL-17, leads to impaired fungal control or even lethal dissemination (Conti et al. 2009; Gladiator et al. 2013; Trautwein-Weidner et al. 2015b; Schönherr et al. 2017; Kashem et al. 2015a). From the fungal side, it was shown that IL-17A directly targets *C. albicans* cells and induces a stress response similar to nutrient starvation (Zelante et al. 2012).

It was shown that neutrophil recruitment and activation during OPC is independent of IL-17. During acute OPC IL-17 induces the production of host defense peptides, especially S100A8/9 and mBD3 (Trautwein-Weidner et al. 2015b). In our study, all isolates triggered a strong IL-17 response, irrespective of their pathogenicity, albeit the IL-17 induction was delayed in case of the persistent isolates. The delayed IL-17 induction did not entail an enhanced neutrophil response at any time. The qualitative differences in the host response between different *C. albicans* isolates, and in particular the observation that the delayed induction of IL-17 by persistent isolates was not accompanied by significant neutrophil infiltration, underlines the notion that the IL-17 and neutrophil pathways are uncoupled during OPC (Trautwein-Weidner et al. 2015b). By testing these strains in OPC in IL-17-deficient mice, we were able to demonstrate that the IL-17 pathway is required for preventing fungal overgrowth irrespectively of the pathogenicity of the strain. Supporting the fact that the IL-17-dependent antimicrobial mechanisms are needed for fungal control. This implies that AMPs or other IL-17 effector molecules might reach the cornified layer of the epithelium where the persistent isolates reside, while neutrophil do not get recruited. Histological analysis revealed that the persistent strains

were restricted to the stratum corneum. Nevertheless, IL-17 and its targets are not sufficient for fungal clearance in case of persistent isolates. In conclusion, for complete fungal clearance of highly pathogenic strains, IL-17 independent neutrophil recruitment and IL-17 dependent antimicrobial effector mechanisms are needed (Trautwein-Weidner et al. 2015b). Whereby neutrophils, IL-17 and induced effector molecules might diminish the fungal outgrowth of low-pathogenic strains but are not sufficient to clear the fungus

In our study we highlighted the key role of the cytokine IL-17 in protective immunity against *C. albicans*. The IL-17 pathway was required for the prevention of fungal outgrowth and to control the infection, irrespective of the pathogenicity of the isolates tested. With this work we raised the importance of the natural diversity of *C. albicans*. The genetic understanding to the phenotypic and functional diversity has to be studied. Our study is the first to show strain dependent host immune responses and persistence in infection *in vivo* and highlights the importance of natural diversity in the research of fungal infections.

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10 Abbreviations

Als	Agglutinin-Like Sequence
APC	antigen presenting cell
APECED	recessive autoimmune polyendocrinopathy syndrome
CARD9	caspase recruitment domain-containing protein 9
CC	clonal Complex
CDC3	cell division cycle protein
CMC	Chronic mucocutaneous candidiasis
CR	complement receptor
DC	Dendritic cell
DNA	deoxyribonucleic acid
Ece1	Extent of Cell Elongation, Candidalysin
EF3	elongation factor 3
EGFR	epidermal growth factor receptor
EV	extracellular vesicle
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	glycophosphatidylinositol
Her2	human epidermal growth factor receptor 2
HIS3	imidazole glycerol phosphate dehydratase
HIV	Human Immunodeficiency Virus
Hst-5	Histatin 5
Hwp1	hyphal wall protein
IL	interleukin
ILC	innate lymphoid cells
LL-37	Cathelicidin
LOH	loss of heterozygosity
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
MAPK	mitogen-activated protein kinase
mBD	murine beta defensin
MLMT	mulitlocus microsatellite typing
MLST	multilocus sequence typing

NET	neutrophil extracellular traps
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
OPC	oropharyngeal candidiasis
PAMP	pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PRR	pathogen recognition receptors
RNA	ribonucleid acid
RVVC	recurrent episodes of vulvovaginal candidiasis
S100A8/S100A9	subunits of calprotectin
SAP	secreted asparty proteinases
spp.	species
ST	sequence type
STAT1/STAT3	Signal transducer and activator of transcription 1/3
Syk	spleen tyrosine kinase
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
VVC	vulvovaginal candidiasis

11 Appendix

11.1 Clinical study: Oral and anal colonisation by *Candida* species in children an open, prospective, non-interventional, matched case study

Servizio di pediatria **Ospedale regionale di Lugano** Via Tesserete 46 6900 Lugano tel. +41 91 811 6835 fax +41 91 811 6836	
Clinical Study Protocol	
Title:	Oral and anal colonisation by *Candida* species in children: an open, prospective, non-interventional, matched case study
Clinical Phase:	
Clinical Monitor:	Franziska Schönherr Istituto cantonale di microbiologia Via Mirasole 22 6501 Bellinzona tel. +41 91 814 60 11 fax +41 91 814 60 19 franziska.schoenherr@biol.ethz.ch
Co-ordinating Investigator:	Dr. med. Valdo Pezzoli
Institute / Departments:	Servizio di Pediatria Ospedale regionale di Lugano Via Tesserete 46, 6900 Lugano Tel: +41 91 811 68 35 Fax: +41 91 811 68 36 valdo.pezzoli@eoc.ch

Status, Version and Date of Protocol:	Version 2 (Amendment 1) Date of protocol: 06February 2014
Planned Dates of Study:	01.03.2013-01.01.2016 First subject in: March 2013; Last subject out: March 2015
Confidential	

Study Synopsis

Name of company: Dipartimento di pediatria Ospedale regionale di Lugano		**Tabulated Study Protocol**	
Protocol date: 11 February 2013	**Trial Number:** **2669**	**Planned Study period:** 01.03.2013-01.01.2016	

Title of study:	Oral and anal colonisation by *Candida* species in children: an open, prospective, non-interventional, matched case study
Investigators:	Dr. med. Valdo Pezzoli
Study centre(s):	4 (Ospedale Regionale di Lugano, FMH medicina intensiva via Morosini 6 6943 Vezia; via Monte Boglia 5 6900 Lugano; Medici-Pediatria via Foletti 19 6900 Massagno)
Clinical phase:	n.a.
Objectives:	To study the colonisation by *Candida* species of the mouth and the anal region of children with recurrent candidiasis and healthy, matched controls.
Methodology:	Open, prospective, non-interventional and non randomised, matched case control study
No. of subjects **total:** **each group:**	 30 15 (20 / 60 will be enrolled to cater for dropout and non-carriers)
Diagnosis and main criteria for inclusion:	Children with recurrent candidiasis and corresponding matched controls with no overt candidiasis Age: 0-12 years
Procedures:	Swabs from skin, throat/mouth and anal region • First swab taken at hospital by treating physician or study nurse • Follow up swabs to be taken by parents during 3 weeks (1 swab/week)
Duration of treatment:	4 weeks

Name of company: Dipartimento di pediatria Ospedale regionale di Lugano		**Tabulated** **Study Protocol**	
Protocol date: 11 February 2013	**Trial Number:** **2669**	**Planned Study period:** 01.03.2013-01.01.2016	

Study Schedule:	Swabs will be taken using cotton swabs by the investigator or a study nurse at the time of diagnosis (in the case of candidiasis patients) or during a regular control visit (for the matched controls)
Criteria for efficacy:	
Criteria for safety:	No safety data will be collected, this being a non-interventional study
Statistical methods:	Pielou's Evenness index for primary endpoint; Richness, Shannon and Simpson diversity indices for the secondary endpoints; contingency tables for frequencies of colonisation; demographic data will be presented using descriptive analyses (mean, standard deviation, 95% confidence intervals, percentages)

Flow chart

Study Periods	***Screening***	***Observational Period***		
Visit	**1**	**2**	**3**	**4**
Follow-up	Day 0	Day 7 +/- 2	Day 14 +/- 2	Day 21 +/- 2
Informed Consent	*x*			
Demographics	*x*			
Medical History	*x*			
In- /Exclusion Criteria	*x*			
Physical Examination	*x*			
Swabs	*x*	*x**	*x**	*x**
Concomitant Therapies	*x*	*x*	*x*	*x*
Conclusion of Subject Participation				

* at home by parents after instruction

Introduction

Species of *Candida* are common commensals of the skin and mucosae of the mouth, gastrointestinal and genital tract in humans. They may cause superficial mucosal and cutaneous diseases, as well as deep tissue and blood infections in humans (1; 2).

It is still unclear why *Candida*, in particular *C. albicans*, can become pathogenic not only in immunosuppressed or debilitated patients, but also in healthy individuals. Published data indicate that 25-35% of women are normal carriers of *Candida*, but not all of them develop infections (3), suggesting that these pathogens may act through a mechanism of immune evasion depending on different virulent factors possibly associated to strain specific features.

The influence of predisposing factors on the colonisation and infection by *Candida* spp. and the outcome of the infection have been studied in clinical settings (4), yet no clear correlation has been established between patients' characteristics and the phenotype/genotype of the etiological agent.

Candida species may colonise the mucosal surface, on which they normally live without causing any symptoms. In some cases, however, they may cause a so-called oral thrush, particularly in infants and young babies. Thrush may occur when the yeast grows particularly well, forming extended colonies. Known predisposing host factors could be the occurrence of an unbalanced mouth flora during antibiotic treatment or a special diet (5; 6).

According to the literature, 5-7% of less than one-month old babies may develop an oral candidiasis. Groups at increased risk are infants, AIDS and cancer patients, where 9-31% may develop an infection with *C. albicans* (7)

Medical Background

Oral candidiasis is caused by an extended growth of *Candida* species. Four main different groups of oral forms are distinguished: pseudomenbranous (thrush), chronic erythematous, acute erythematous, and chronic hyperplastic candidiasis (6).

50% of oral candidiasis cases are caused by *Candida albicans* (5). Especially imunocompromised patients can develop a chronic pseudomembranous candidiasis, which can be treated, but often the infection is recurrent (8).

Competition among bacteria and fungi is very extensive on mucosal surfaces, because the nutrients are limited; this provides a selective pressure. The nutritional competition, the elaboration of toxic and

metabolic products and the alteration of the microenvironment are part of microbial interactions. The native bacterial flora can decrease colonization of *Candida albicans* by competition (8).

So far, nothing is known on the genotypic composition of the *Candida* populations colonising the mouth and anal region of children with a thrush, as opposed to healthy controls. It is also unknown whether or not *Candida* strains colonising children with candidiasis are genotypically substantially different from those present on healthy controls.

Description and Rationale for Performing the Trial

The project presented here is the result of collaborative efforts between the paediatrician Dr. med. Valdo Pezzoli (Ospedale civico di Lugano) and the Cantonal institute of microbiology (ICM Bellinzona) and.

This project has mainly an epidemiological value and the data resulting from this work will be evaluated with the aim of collecting information that may later be used to plan a larger work on the epidemiology and distribution of *Candida* species in small children, as well as of understanding the genotypic composition of Candida populations in diseased and healthy children, but the strains isolated will also be used within the scope of a larger project that aims at elucidating the pathogenicity of *Candida*.

We shall, in fact, use the *C. albicans* isolated from symptomatic children and healthy matched controls to identify biomarkers that could distinguish pathogenic *C. albicans* from commensal *C. albicans*, because in a pilot study we observed that some pathogenic strains were significantly more resistant to at least two oxidant agents as compared with isolates from healthy volunteers. Within the scope of the same project, we should also like to study changes on protein expression in *C. albicans* as a response to several physiological conditions. Identification of valid biomarkers will help to clarify the complex nature of the fungus-host interaction. We should then like to characterise immunological parameters of the patients for which clinical pathology and physiology data are available, as well as clinical risk factors that could be responsible for the outcome of the fungal infection, and to correlate them with the results of the molecular, biochemical and proteomic characterisation.

Benefit / Risk Assessment

The number of patients who develop fungal infections has increased dramatically in the last decades. Candidiasis rates are still extremely high, particularly in immunosuppressed patients. A more detailed knowledge of the epidemiology of *Candida* species in children will be helpful to predict infection patterns and eventually to develop effective prevention or/and treatment schedules of oral thrush. The genotypic and phenotypic characterization of the fungal strains isolated will additionally allow us

to prove or dispel the generally accepted dogma that infections by *Candida* are driven only by the patient's predisposition. The medical information we can obtain from this study may also allow us to better characterize the immune response to *Candida* infections by relating the genotype of the isolated strains with the type of patient/volunteer colonised. The results collected will have obvious advantages for the planning of therapeutic interventions against candidiasis. The possibility of studying molecular pathways possibly altered after fungal infections in cells of the immune system will be of great interest to clarify the complicated scenario occurring during fungal infection.

This being a non-interventional, non-invasive trial, no risks are foreseen for the participating children, so that the benefits of the trials will clearly outweigh any risk.

Study Objectives

General Aim / Primary Objective

To investigate the epidemiology of *Candida* strains in oral candidiasis patients and to compare the taxonomic and genetic composition of the fungal (*Candida*) biota of children with recurrent candidiasis with that of matched case controls.

Primary Endpoint

The diversity of the *Candida* populations isolated from the mouth of the patients suffering from thrush and from the healthy controls as expressed by the Pielou's Evenness Index.

Secondary Endpoints

The diversity of the *Candida* populations isolated from the mouth of the patients suffering from thrush and from the healthy controls as expressed by the Pielou's Evenness Index and the Shannon and Simpson Diversity Indices.

The diversity of the *Candida* populations isolated from the anal regions of the patients suffering from thrush and from the healthy controls as expressed by the Pielou's Evenness Index and the Shannon and Simpson Diversity Indices.

The colonisation rates by different *Candida* species in the oral and anal regions between children suffering from recurrent candidiasis as compared to children with no overt candidiasis but with *Candida* colonisation.

The diversity of the *Candida* populations isolated from the skin of the patients suffering from thrush or from the healthy controls as expressed by the Pielou's Evenness Index and the Shannon and Simpson Diversity Indices.

Determination of the gene expression and proteome profiles of the isolates obtained during the study and the correlation of their pathogenicity with the health status of the children as described by their general medical history.

The diversity of the *Candida* populations isolated from the mouth of the patients suffering from thrush after antimycotic treatment as expressed by the Pielou's Evenness Index and the Shannon and Simpson Diversity Indices.

Description of design and trial population

Overall Study Design and Plan

Open, prospective, non-interventional and non randomised, matched case study.

Discussion of study design, including the choice of control groups

The design of this study is appropriate to identify the differences in colonisation by different *Candida* species in a population of children with recurrent oral candidiasis (oral thrush). The use of a matched case control design will allow us to get first information on the conditions that may be responsible for the development of a thrush and to get a reliable estimate of the sample size to be used in a follow-up, main study.

Selection of Study Population

The study population will consist of children between 0-12 years with oral recurrent candidiasis.

The case controls will be determined by age, race, and gender. The match case control should also have a colonization by *Candida* but no symptoms.

Inclusion Criteria

- Age between 0-12 years
- Informed consent signed
- No immunosuppression or immune diseases
- Presence of a candidiasis (in the candidiasis group) or symptomless *Candida* colonisation in the control group.

- History of candidiasis (at least one oral thrush before the screening visit) in the candidiasis group.

Exclusion Criteria

- No colonization by *Candida* in the matched controls.
- Current intake of antibiotics
- Current participation in another clinical study or having already been enrolled in this study.

Primary Efficacy Variables.

The diversity of the *Candida* populations isolated from the mouth of the patients suffering from thrush and from the healthy controls as expressed by the Pielou's Evenness Index.

Secondary Efficacy Variables

The diversity of the *Candida* populations isolated from the mouth of the patients suffering from thrush and from the healthy controls as expressed by the Pielou's Evenness Index and the Shannon and Simpson Diversity Indices.

The diversity of the *Candida* populations isolated from the anal regions of the patients suffering from thrush and from the healthy controls as expressed by the Pielou's Evenness Index and the Shannon and Simpson Diversity Indices.

The diversity of the *Candida* populations isolated from the skin of the patients suffering from thrush or from the healthy controls as expressed by the Pielou's Evenness Index and the Shannon and Simpson Diversity Indices.

The colonisation rates by different *Candida* species in the oral and anal regions between children suffering from recurrent candidiasis as compared to children with no overt candidiasis but with *Candida* colonisation.

Determination of the gene expression and proteome profiles of the isolates obtained during the study and the correlation of their pathogenicity with the health status of the children as described by their general medical history.

The diversity of the *Candida* populations isolated from the mouth of the patients suffering from thrush after antimycotic treatment as expressed by the Pielou's Evenness Index and the Shannon and Simpson Diversity Indices.

Appropriateness of Measurements and Variables

The data collected are appropriate for an epidemiological study of *Candida* colonisation and infection in children. All variables are well suited for the evaluation by the diversity indices foreseen and contingency tables as usual in epidemiological studies.

Routine Monitoring

Case report forms, progress notes and copies of laboratory and medical test results must be available at all times for inspection by the sponsor's clinical study monitor, the health authorities (e.g. FDA) and the IRB/IEC. The field monitor may review (all or in part) case report forms, and written informed consents. The accuracy of the data will be verified by reviewing the above referenced documents.

Audits and Inspections

A quality assurance audit/inspection of this study may be conducted by the sponsor/regulatory authorities/IRB/IEC, respectively. The quality assurance auditor/inspector will have access to all medical records, the investigator's study related files and correspondence, and the informed consent documentation that is relevant to this clinical study.

Investigational Plan

For children with recurrent candidiasis:

The first visit at the hospital for a recurrent candidiasis will be the first time point of the observational period. The parents, if agreeable to have their child participate in the investigation, will have to sign the informed consent; demographics and medical history will be recorded in the case report form (CRF). The paediatrician will also record any concomitant therapy. After checking of the In/Exclusion Criteria a physical examination will be carried out and swabs from the mouth region/throat, anal regions and if appropriate of skin will be taken by the treating physician or a study nurse. Samples 2-4 will be taken at home by the parents at weekly intervals, after careful instruction of the parents by the physician on how to take the swabs and send them to the laboratory.

For healthy volunteers (matched case controls):

During a control visit of their child, the parents will be asked to participate in the study. This control visit coincides with the Screening visit and, if agreeable to have their child take part in the investigation, the parents will be asked to fill in the informed consent. Demographics, medical history and concomitant therapies will be recorded. After checking of the In/Exclusion Criteria a physical

examination will be carried out and swabs from mouth region/throat, anal regions and if appropriate of the skin will be taken by the treating physician or a study nurse. The swab(s) will be immediately plated out in the lab and the culture results communicated to the physician, who will then inform the parents about the continuation of the trial. If the swab is positive for Candida species and the In/Exclusion Criteria are fulfilled, the child will be included in the study. Samples 2-4 will be taken at home by the parents at weekly intervals, after careful instruction of the parents by the physician on how to take the swabs and send them to the laboratory.

Trial procedures at each visit

Screening Phase

For all participants the screening phase is also the first time point of the observational period.

Observational period

The observational period is 4 weeks and samples will be taken at weekly intervals. Sample #1 will be taken in the hospital. Samples #2 to #4 will be taken by the parents and sent directly to the laboratory in Bellinzona.

Follow-up and end of trial

No follow-up period is foreseen.

Molecular analyses

All samples will be studied using standard molecular and biochemical techniques, which include microsatellite sequencing, biofilm formation, growth on menadione and other techniques that the biologists may deem appropriate to differentiate genotypically and biochemically the strains collected.

Removal of Patients from Therapy or Assessment (Adherence to Protocol)

Dropouts and Withdrawals

Any dropouts will be replaced until the final number of 15 participants per group will be reached.

Statistical Methods and Determination of sample size

Statistical Design/Model

This study has been conceived as a pilot investigation, because no data are available in the literature to determine the sample size needed to provide reliable epidemiological data on candida colonisation

in patients with recurrent candidiasis and healthy controls. Thus, no statistical design has been set up for the study. All analyses will be carried out on an exploratory basis only.

Null and Alternative Hypotheses

Primary endpoint

H_0: No difference exists in the diversity of the *Candida* populations isolated from the mouth of the patients suffering from thrush and from the healthy controls as expressed by the Pielou's Evenness Index.

H_1: There is a difference in the diversity of the *Candida* populations isolated from the mouth of the patients suffering from thrush and from the healthy controls as expressed by the Pielou's Evenness Index.

Secondary endpoint

The null hypotheses for the secondary endpoints will be constructed as for the primary endpoint.

Planned Analyses

This being a pilot trial, no specific statistical analyses have been planned. Data will be presented and analysed by chi-square for the contingency tables in the case of frequency rates and by appropriate tests for diversity indices, using the available software (e.g. http://www.ecosym.univ-montp2.fr/). Demographic data will be presented using standard parametric and non-parametric measures.

Interim Analyses

No interim analyses are foreseen.

Handling of Missing Data

No plans for handling dropouts and imputing missing data are foreseen. Enrolment will be continued until the planned number of 15 subjects per group will be reached.

Evaluable subjects are thus all subjects that provide data for evaluation at all five sampling time points.

Randomisation

No randomisation of the subjects is foreseen.

Determination of Sample Size

The sample size of 30 subjects is not based on any statistical considerations, but rather on the maximum number of samples that can be reasonably worked up in the lab by the existing manpower. On the other hand, 15 subjects for each group should yield a sample size large enough to achieve the objectives of the study. Considering an estimated dropout rate of 20%, the inclusion of 20 subjects in the candidiasis group should be enough to reach the planned sample size of 15.

For the matched case controls one can expects approximately 30% of the subject to have a symptomless colonisation by *Candida* species. Thus we plan to screen 50 children to have at least 15 healthy volunteers colonised by *Candida*. Considering again an estimated dropout rate of 20%, the inclusion of 60 subjects in this group should be enough to reach the planned sample size of 15.

Administrative matters

Ethics

The trial will be carried out in compliance with the protocol, the principles laid down in the Declaration of Helsinki, latest version, in accordance with the ICH Harmonised Tripartite Guideline for Good Clinical Practice (GCP) and in accordance with applicable regulatory requirements.

Institutional Review Board (IRB) or Independent Ethics Committee (IEC)

According to the regulatory requirements of the participating country, the study will not start before the protocol has either been reviewed or approved by the local institutional review board (IRB) i.e. an Independent Ethics Committee (IEC). The constitution of the IRB or IEC must meet the requirements of the participating country. The IRB or IEC must also perform all duties outlined by the requirements of the participating country.

Patient Information and Informed Consent

Prior to subject participation in the study, informed consent will be obtained from each subject (or the subject's legally authorised representative) according to the Swiss regulatory and legal requirements. This consent must be personally dated and signed by the subject (or its legally authorised representative) and the investigator (or his designee) and retained by the investigator as part of the study records. The subject must be informed that his / her medical records may be examined by authorised representatives from ICM, of health authorities and IRB/IECs. Should a protocol addendum (a)/amendment be made, the subject consent form may be revised to reflect the changes of the protocol. It is the responsibility of the investigator to ensure that an amended consent is approved or

reviewed by the IRB or the IEC, and that it is signed by all subjects subsequently entered in the study and those currently in the study, if affected by the amendment.

Records

Case Report Forms

Case report forms for individual subjects will be provided by the sponsor. A copy will be given to the laboratory, the original will remain with the investigator. Case report forms are used to record clinical study data and are an integral part of the study and subsequent reports. The case report forms, therefore, must be legible and complete. All forms should be filled using a black ball point pen. Errors should be lined out but not obliterated and the correction inserted, initialled and dated. A declaration ensuring accuracy of data recorded in the case report forms must be signed by the investigator. Case report forms must be kept current to reflect subject status at each phase during the course of study. Subjects are not to be identified on the case report form by name. Appropriate coded identification (e.g. Subject Number) and subject initials must be used. Documented medical histories and narrative statements relative to the subject's progress during the study will be maintained. These records should also include originals or copies of laboratory and other medical test results (e.g. ECGs, etc.), which must be kept on file with the individual subject's case report forms. The investigator will be responsible for retaining all records pertaining to the study in accordance with local regulatory and legal guidelines.

Source documents

Source documents provide evidence for the existence of the subject and substantiate the integrity of the data collected. Source documents are filed at the investigator’s site. Data reported on the Case Report Forms that are derived from source documents must be consistent with the source documents or the discrepancies must be explained.

The investigator may need to request previous medical records or transfer records, depending on the trial; also current medical records – not just shadow charts – must be available.

The following data to be reported on the CRF should be included and derived from the source documents:

Subject identification (gender, data of birth)

Subject participation in the trial (substance, trial number, subject number, date informed consent given)

- Dates of subject's visits
- Medical history
- Medication history
- Conclusion of subject's participation in the trial.
- No AE data will be collected, this being no interventional trial.

Direct access to source data/documents

The investigator / institution will permit trial related monitoring, audits, IRB / IEC review and regulatory inspection, providing direct access to all related source data / documents. Case report forms and all source documents, including progress notes and copies of laboratory and medical test results must be available at all times for review by the sponsor's clinical trial monitor and inspection by health authorities (e.g., Swissmedic). The on-site monitor may review all case report forms, and written informed consents. The accuracy of the data will be verified by reviewing the documents described in Section 7.2.2.

Quality Assurance Audit

A quality assurance audit of this trial may be conducted by the sponsor or sponsor's designees. The quality assurance auditor will have access to all medical records, the investigator's trial related files and correspondence, and the informed consent documentation that is relevant to this clinical trial.

Rules for amending protocol

All amendments must be documented, dated and signed by the investigator and sponsor.

Discontinuation of the study

ICM and the investigator reserve the right to discontinue this study at any time for failure to meet expected enrolment goals and any other administrative reasons.

Statement of confidentiality

Individual subject medical information obtained as a result of this study is considered confidential and disclosure to third parties is prohibited. Subject confidentiality will be further ensured by utilising subject identification code numbers to correspond to treatment data in the computer files. Such medical information may be given to the subject's personal physician or to other appropriate medical personnel responsible for the subject's welfare. Data generated as a result of this study are to be

available for inspection on request by the participating physicians, the sponsor's monitors, by the IRB or IEC and the regulatory health authorities.

Insurance

The study is performed within the scope of clinical activities covered by the insurance of civil liability of EOC.

REFERENCES

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12 Curriculum Vitae

Personal information

Name:	Franziska Annett SCHÖNHERR
Date of birth	20.02.1988
Citizenship	German
Main residence	Riedtlistrasse 7, 8006 Zurich
Mail adress	franzi.schoenherr@gmail.com
Mobil	+4178 644 70 24

Education and Experience

10/2012-12/2016 **ETH Zurich (10/12-04/15) and University of Zurich (04/15-12/16)**

PhD thesis on host-pathogen interactions, functional and genetic studies on the yeast *Candida albicans*

member of the Life science graduate school

group leader: Prof. Salomé Leibundgut-Landmann

external dissertation at the Laboratory of Applied Microbiology, Ticino, CH (form. Istituto cantonale di Microbiologia Bellinzona)

- Worked in a multidisiplinary team between three institutions in Switzerland
- Established several international collaborations
- Supervision of bachelor, master students
- organization and teaching in several block courses in microbiology and immunology
- Clinical monitor of a local clinical trial on the oral and anal colonisation of *Candida* species in healthy children

06/2012-07/2012 **Ernst-Moritz-Arndt University Greifswald**

Research assistant

project: Pathophysiology in *Staphylococci* in the post-genomic era

(Prof. Michael Hecker, Dr. Ulf Gerth)

- Supervision of two bachelor students

10/2006-04/2012 **Biology study at the Ernst-Moritz-Arndt University of Greifswald**

main subject: microbial physiology and molecular biology

	minor subjects: biochemistry, genetics
	Diploma thesis: protein synthesis and degradation in glucose-starved *Staphylococcus aureus*
	final grade: 1,6 (1=very good; 6=insufficient)
01/2010-03/2010	**internship at the University of Copenhagen**
	Department of veterinary disease biology, Section Microbiology
	(Prof. Hanne Ingmer, Dr. Dorte Frees)
08/2009-01/2010	**semester abroad at the University of Copenhagen**
	Faculty of Life Science
1998-2006	**academic high school: Gymnasium Marienberg**
	main subjects: biology, german
	final grade: 2,2
1994-1998	**grammar school: Gerhardt Hauptmann Grundschule Lauterbach**

Further education

- Introductory course on animal experiments
- Project management for research
- Voice training and presentation skills
- Advanced Proteomics course

Awards

04/2016	Travel grant ASM, USA
02/2016	Travel grant LSZG Zurich
07/ 2016	Travel grant SGM/SSM Switzerland
08/2015	Best oral presentation
	Retreat of the graduate school Microbiology&Immunology
	Title: Fungal determinants govern the host response to *Candida albicans* in the oral mucosa
10/2014	Travel grant SGM/SSM Switzerland

Conferences and Meetings attended

Friday noon seminar series, 2016, Zurich (CH)
(invited speaker)

SSM/SGM (Swiss Society for Micorbiology), 2016, Bern (CH)
(oral and poster presentation)

ASM (American society for microbiology) Candida and Candidiasis

2015, Seattle (USA)
(oral and poster presentation)

SSM/SGM (Swiss Society for Micorbiology), 2015, Lugano (CH)
(poster presentation)

VAAM (Association for general and applied microbiology), 2014
Dresden (GER)

Trends in Medical Mycology, 2014, Copenhagen (DK)
(poster presentation)

SSM/SGM (Swiss Society for Micorbiology), 2013, Interlaken (CH)
(poster presentation)

language skills

German	native language
English	fluent
Danish	basic knowledge
Italian	basic knwoledge

memberships

Swiss Society of Microbiology

American Society of Microbiology

Life science graduate school

13 Acknowledgment

"The strength of the team is each individual member.

The strength of each member is the team." P. Jackson

Several people contributed in some way to this PhD thesis, which I would like to thank sincerely:
First of all, I would like to thank my PhD supervisor Salomé LeibundGut-Landmann for your ideas, your enthusiasm, endless input to my projects and the support. I could always rely on your help, even when we were separated by the gotthard massif most of the time.
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